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**Steven M. BESSETTE, et al.**

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## FOREIGN PATENT DOCUMENTS

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## REPORTS

**Monoterpenes Inhibit Cell Growth, Cell Cycle Progression, and Cyclin D1 Gene Expression in Human Breast Cancer Cell Lines**

Sylvie Bardon, Karine Picard, and Paule Martel

**Abstract:** Monoterpenes are found in the essential oils of many commonly consumed fruits and vegetables. These compounds have been shown to exert chemopreventive and chemotherapeutic activities in mammary tumor models and represent a new class of breast cancer therapeutic agents. In this study, we investigated the effects of limonene and limonene-related monoterpenes, perillyl alcohol and perillic acid, on cell growth, cell cycle progression, and expression of cyclin D1 cell cycle-regulatory gene in T-47D, MCF-7, and MDA-MB-231 breast cancer cell lines. Our results revealed that limonene-related monoterpenes caused a dose-dependent inhibition of cell proliferation. Of the three monoterpenes tested, perillyl alcohol was the most potent and limonene was the least potent inhibitor of cell growth. The enantiomeric composition of limonene and perillyl alcohol did not interfere with their effect on cell growth. Sensitivity of breast cancer cell lines to monoterpenes was in the following order: T-47D > MCF-7 > MDA-MB-231. Growth inhibition induced by perillyl alcohol and perillic acid was associated with a fall in the proportion of cells in the S phase and an accumulation of cells in the G<sub>1</sub> phase of the cell cycle. Finally, we showed that the effects of limonene-related monoterpenes on cell proliferation and cell cycle progression were preceded by a decrease in cyclin D1 mRNA levels.

**Introduction**

Monoterpenes are natural plant products found in the essential oils of many commonly consumed fruits and vegetables. For nearly 50 years, they have been widely used as flavor and fragrance additives in food and beverages. A number of recent studies have shown that monoterpenes possess antitumorigenic activities and suggest that these compounds represent a new class of agents for cancer

chemoprevention (1,2). Limonene, the simplest and most frequently occurring monocyclic monoterpene, and perillyl alcohol, a hydroxylated limonene analog, have demonstrated chemopreventive and chemotherapeutic activity against a variety of organ-specific cancers in rodent models, including skin, lung, pancreas, colon, and mammary cancers (2-5). Dietary administration of limonene or perillyl alcohol can inhibit tumor formation and cause complete regression of early or advanced chemically induced mammary carcinomas (6,7). A 7.5% dietary level of limonene is required to induce tumor regression, whereas perillyl alcohol has been shown to be 5-10 times more potent than limonene at inducing tumor regression (7). Limonene and perillyl alcohol are rapidly metabolized in a similar manner by humans and rats, and their major circulating metabolite is perillic acid (2,7). Monoterpenes do not cause systemic toxicity at the doses required to induce regression of mammary carcinomas. On the basis of these data, monoterpenes are currently being tested in phase I clinical trials on advanced cancer patients in the United Kingdom (8) and the United States (9).

Antiproliferative effects of limonene, perillyl alcohol, and their metabolite perillic acid have been previously reported *in vitro* in several cell lines, including NIH/3T3 rat fibroblasts, HT-29 human colon carcinoma cells, and PANC-1 human pancreas carcinoma cells (10,11). However, the direct effects of monoterpenes on breast cancer cells have not been described.

The present study was designed to investigate the effects of monoterpenes in three human breast cancer cell lines, T-47D, MCF-7, and MDA-MB-231. We evaluated the effects of monoterpenes on cell proliferation, cell cycle progression, and expression of the oncogene cyclin D1, which is involved in the regulation of the cell cycle and often amplified or overexpressed in breast carcinomas (12,13). We compared the effects of limonene, perillyl alcohol, and perillic acid.

## Materials and Methods

### Monoterpenes

The two optically active forms (*R* and *S*) of limonene and perillyl alcohol were purchased from Sigma-Aldrich Chemical (St. Quentin Fallavier, France). (*S*)-perillic acid came from Extra-Synthese (Genay, France).

### Cell Lines and Culture Conditions

T-47D cells were obtained from American Type Culture Collection (Rockville, MD); MCF-7 and MDA-MB-231 cell lines were kindly provided by Dr. Françoise Vignon (INSERM U148, Montpellier, France). T-47D cells were routinely cultured in RPMI medium, MCF-7 in 1:1 Dulbecco's modified Eagle's medium-F-12 medium, and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (GIBCO BRL, Life Technologies, Cergy-Pontoise, France). All cell lines were maintained in medium supplemented with 10% fetal calf serum (FCS), penicillin (25 U/ml), and streptomycin (25 µg/ml) (GIBCO BRL). Cells growing as monolayers were kept at 37°C in a humid atmosphere in the presence of 5% CO<sub>2</sub>.

### Cell Growth Experiments

Cells were plated on 15-mm-diameter wells (24-well plates, ICN Linbro, Orsay, France) at a density of  $2.5 \times 10^4$  cells/well in 3% FCS-supplemented medium. One day after plating, monoterpenes dissolved in ethanol at 1,000-fold final concentration were diluted with culture medium and added to cells. Control wells were treated with ethanol to the same final concentration. Medium was changed every two days. At the end of the treatment, cells were rinsed with a phosphate-buffered saline solution and fixed with methanol. The amount of cellular DNA was evaluated using the 3,5-diaminobenzoic acid dihydrochloride fluorometric assay (Sigma Chemical) (14). DNA content (mean  $\pm$  SD) was calculated from triplicate wells.

### Cell Cycle Analysis

Near-confluent T-47D cells were treated with 0.5 mM perillyl alcohol, 1 mM perillic acid, or ethanol vehicle for 48 hours in 5% FCS-supplemented medium. Cells were collected by trypsinization, washed two times in cold phosphate-buffered saline, and fixed in cold 66% ethanol. Cells (10<sup>6</sup>/ml) were then permeabilized and stained with a propidium iodide solution (Coulter DNA-PREP reagents, Coultronics, Margency, France). Flow cytometric analysis of stained cells was performed on a Coulter Elite ESP flow cytometer (Coultronics), and histograms were analyzed using Multicycle AV software (Phoenix Flow Systems).

## RNA Isolation and Northern Blot Analysis

Monoterpenes were added to cells in exponential growth. After six hours of treatment, RNA was extracted from triplicate 60-mm cell culture dishes using guanidine isothiocyanate, according to the procedure described by Chomczynski and Sacchi (15). The concentration of RNA was determined spectrophotometrically at 260 nm. Thirty micrograms of total RNA were loaded on a 1% agarose-formaldehyde gel and transferred to nylon Hybond N<sup>+</sup> filters (Amersham, Les Ulis, France). The blots were then hybridized with human cyclin D1 cDNA probe (obtained from Dr. David Beach, Cold Spring Harbor, NY) (16) radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham) by random primer extension (Stratagene multiprime DNA labeling system, Ozyme, Montigny le Bretonneux, France). Cyclin D1 mRNA abundance was quantitated by electronic autoradiography (Packard Instant Imager). The data were expressed in arbitrary units, and all values were standardized with levels of 18S rRNA.

### Statistics

Values are means  $\pm$  SD. Statistical significance was determined using Student's *t*-test, with *p* < 0.05 evaluated as statistically significant.

## Results

### Comparative Effects of Monoterpenes on the Growth of Three Human Breast Cancer Cell Lines

Limonene and perillyl alcohol occur naturally in the (*R*) and (*S*) optically active forms and as (*R-S*) mixtures. We compared the effects of (*R*)- and (*S*)-limonene, (*R*)- and (*S*)-perillyl alcohol, and (*S*)-perillic acid on the growth of T-47D, MCF-7, and MDA-MB-231 cell lines for six days. A range of monoterpene concentrations from 10 µM to 3 mM was tested (Figure 1).

The minimum concentration of (*R*)- and (*S*)-limonene that elicited a significant decrease (15%) in the growth rate of T-47D cells was 3 mM, the highest concentration tested (Figure 1). MCF-7 and MDA-MB-231 cell proliferation was not affected by limonene. No difference was noted between the effects of the two optical forms of limonene.

Both enantiomers of perillyl alcohol markedly inhibited breast cancer cell growth in a dose-dependent manner (Figure 1). A significant inhibition of cell proliferation was seen with (*R*)- or (*S*)-perillyl alcohol from 10 µM in T47D cells and from 0.1 mM in MCF-7 and MDA-MB-231 cells. (*R*)- and (*S*)-perillyl alcohol produced a 90% inhibition of growth at 1 mM in T-47D and MCF-7 cell lines, whereas 3 mM was necessary to attain the same growth inhibition in MDA-MB-231 cells. In the three cell lines the antiproliferative effect of (*R*)-perillyl alcohol was identical to that of (*S*)-perillyl alcohol. Therefore, the following experiments were carried out with (*S*)-perillyl alcohol only. Figure 2 shows the time course of the effects of perillyl alcohol. After three days of treatment,

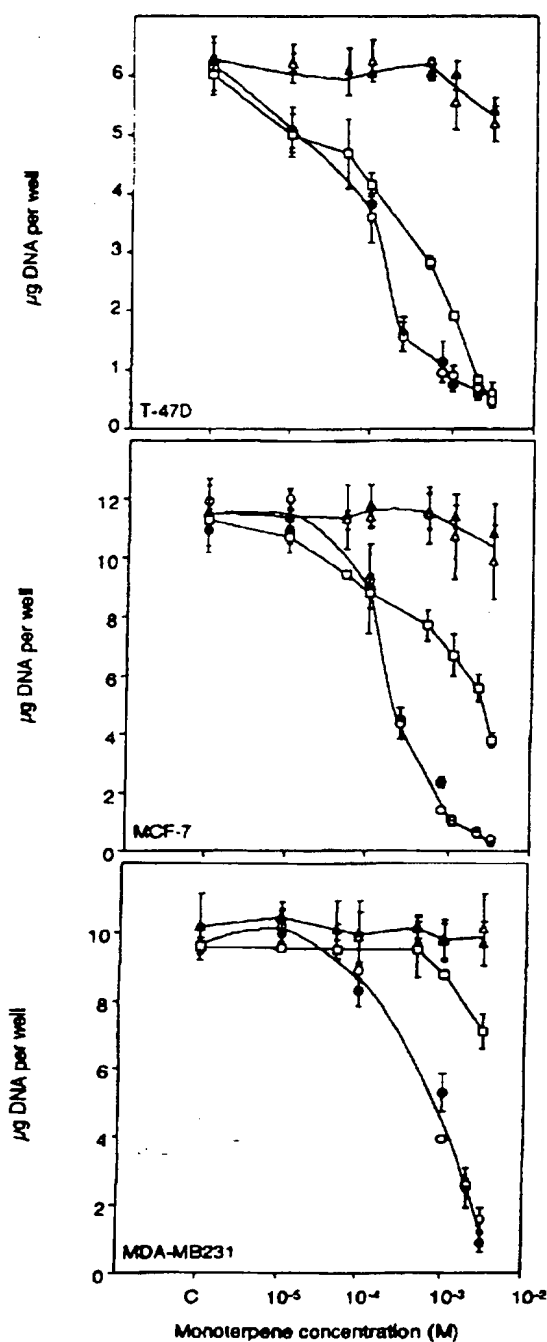


Figure 1. Dose response of breast cancer cell proliferation to monoterpenes. T-47D, MCF-7, and MDA-MB-231 cells were exposed for 6 days to increasing concentrations of (*R*)-limonene (filled triangles), (*S*)-limonene (open triangles), (*R*)-perillyl alcohol (filled circles), (*S*)-perillyl alcohol (open circles), or (*S*)-perillic acid (open squares). Controls (C) were treated with ethanol alone. At end of treatment, DNA content was evaluated using fluorescence diaminobenzoic acid assay on triplicate wells for each determination. Values are means  $\pm$  SD.

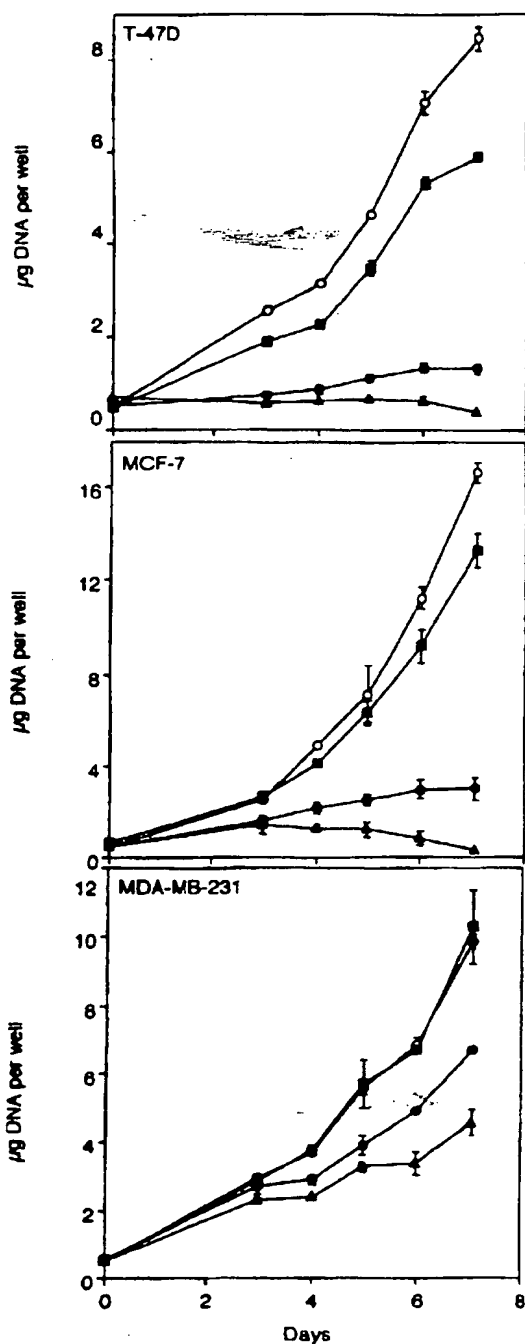


Figure 2. Growth curves of breast cancer cells cultured in presence of perillyl alcohol. T-47D, MCF-7, and MDA-MB-231 cells were treated 7, 6, 5, 4, and 3 days before DNA assay with 0.1 mM (filled squares), 0.5 mM (filled circles), and 1 mM (filled triangles) perillyl alcohol or with solvent alone (open circles). Values are means  $\pm$  SD of 3 determinations.

0.1 mM perillyl alcohol exerted a significant antiproliferative effect in T-47D cells, whereas six days were necessary to obtain a similar effect in MCF-7 cells. However, in both cell lines, 1 mM perillyl alcohol had a cytotoxic effect after seven days of treatment. The MDA-MB-231 cell line was considerably less sensitive to the growth-inhibitory effects of perillyl alcohol, which produced only a 55% growth inhibition at 1 mM after seven days of treatment.

Perillic acid inhibited breast cancer cell proliferation in a concentration-dependent manner, as shown in Figure 1. The inhibition of T47-D cell proliferation was significant from 10  $\mu$ M (17% inhibition) and reached 90% at 3 mM. To a lesser extent, perillic acid affected MCF-7 cell growth, which was reduced by 16% at 50  $\mu$ M and by 66% at 3 mM. MDA-MB-231 cell growth was only slightly affected by perillic acid, which reduced cell proliferation by 26% at a concentration of 3 mM. The time course study presented in Figure 3 showed that 1 mM perillic acid significantly inhibited T-47D cell proliferation from Day 3 of treatment (38% inhibition), whereas five days were necessary to attain a similar degree of inhibition in MCF-7 cells. After seven days of treatment, 1 mM perillic acid reduced cell growth rate by 70% in T-47D cells, 35% in MCF-7 cells, and 17% in MDA-MB-231. We observed that 3 mM perillic acid exerted a cytostatic effect on T-47D cells during the seven days of study. MCF-7 cell growth was markedly inhibited by 3 mM perillic acid but was not totally arrested, with a 70% inhibition at Day 7 of treatment. In the least-sensitive MDA-MB-231 cells, a maximum decrease in proliferation of 32% was observed after seven days of treatment with 3 mM perillic acid.

#### Effects of Monoterpenes on Cell Cycle Phase Distribution

To further elucidate the effects of monoterpenes on breast cancer cell growth, the distribution of T-47D cells between the  $G_0$ - $G_1$ , S, and  $G_2$  + M phases of the cell cycle was recorded after 48 hours of treatment with 0.5 mM perillyl alcohol or 1 mM perillic acid. Figure 4 shows that perillyl alcohol and perillic acid induced an accumulation of T-47D cells in the  $G_1$  phase. This increase was associated with a dramatic decrease in the percentage of S phase cells (>65% inhibition) and, to a lesser extent, in the proportion of cells in the  $G_2$  + M phase (26–35% inhibition). Perillyl alcohol at 0.5 mM was slightly more effective than 1 mM perillic acid to induce changes in T-47D cell cycle phase distribution.

#### Effects of Monoterpenes on Cyclin D1 Gene Expression

Changes in the rate of breast cancer cell cycle progression are often associated with changes in cyclin D1 gene expression (17). To provide some insight into the possible effect of monoterpenes on cyclin D1 gene expression, we carried out Northern blot analysis on total RNA from T-47D and MCF-7 cells treated with 0.5 mM perillyl alcohol or 1 mM perillic acid and from MDA-MB-231 cells treated with 1 mM perillyl alcohol or 3 mM perillic acid. Figure 5 shows

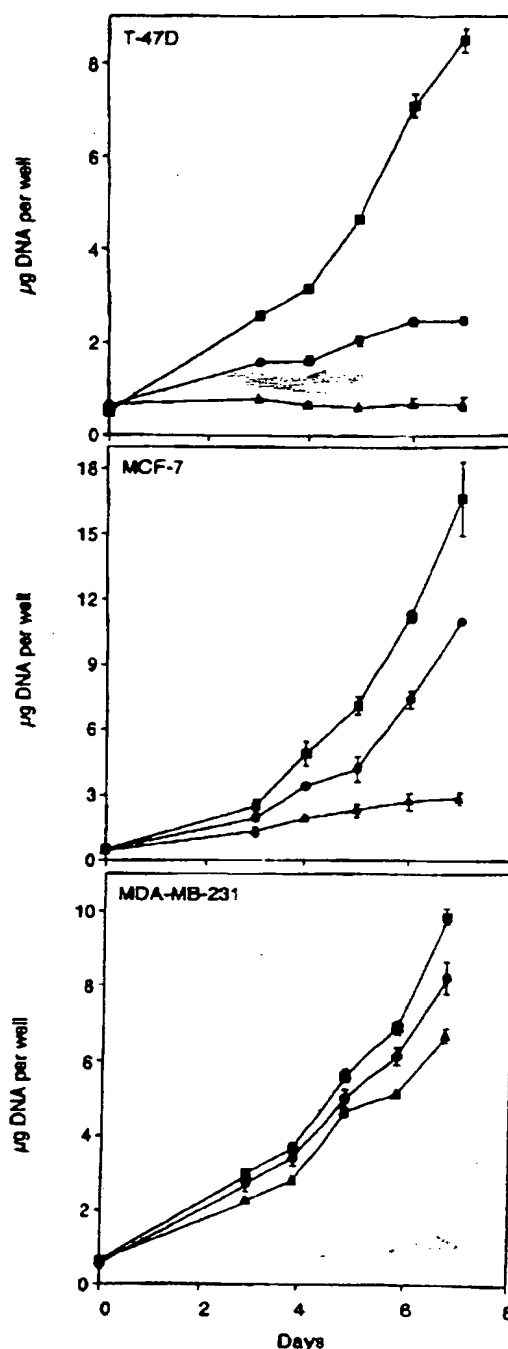


Figure 3. Growth curves of breast cancer cells cultured in presence of perillic acid. T-47D, MCF-7, and MDA-MB-231 cells were treated 7, 6, 5, 4, and 3 days before DNA assay with 1 mM (filled circles) and 3 mM (filled triangles) perillic acid or with solvent alone (filled squares). Values are means  $\pm$  SD of 3 determinations.

that, in the three cell lines, monoterpene treatment resulted in a reduction in cyclin D1 mRNA levels. This effect was observed within 6 hours of treatment with monoterpenes and remained constant over 24 hours (not shown).

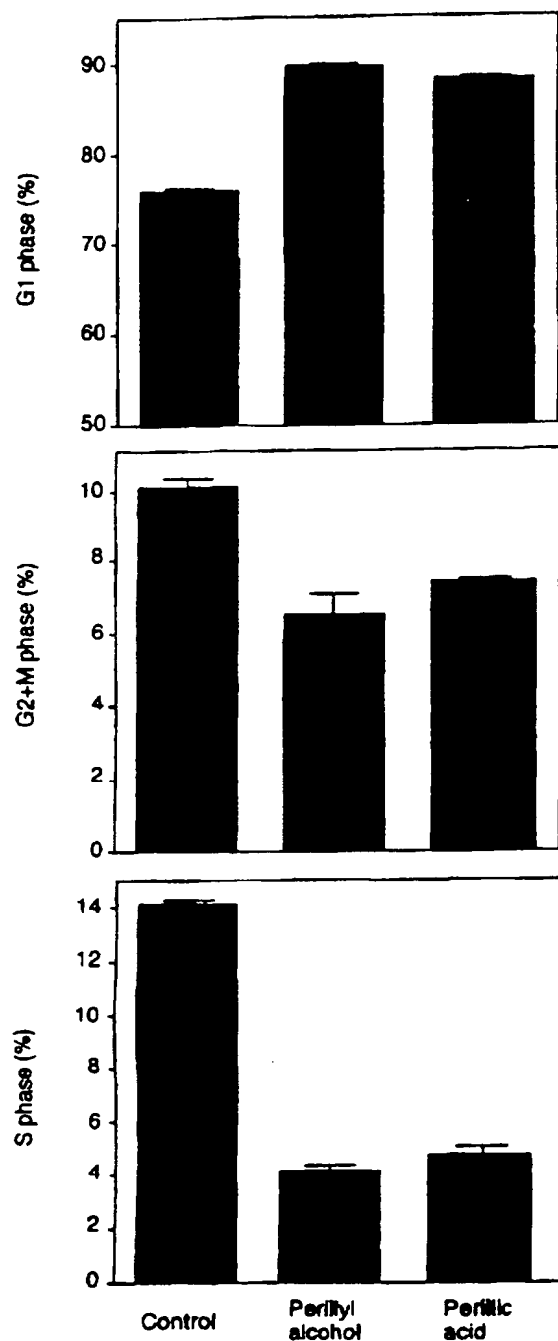


Figure 4. Changes in T-47D cell cycle phase distribution after monoterpene treatment. T-47D cells were treated with perillyl alcohol (0.5 mM), perillic acid (1 mM), or solvent alone and harvested after 48 h for determination of cell cycle phase distribution by flow cytometry. Values are means  $\pm$  SD.

#### Discussion

Monoterpenes represent a new class of naturally occurring therapeutic agents against breast cancer. Limonene, the

simplest monocyclic monoterpene, and perillyl alcohol, a hydroxylated limonene analog, are capable of increasing tumor latency, decreasing tumor multiplicity, and causing regression of rat mammary carcinomas (2,6,7).

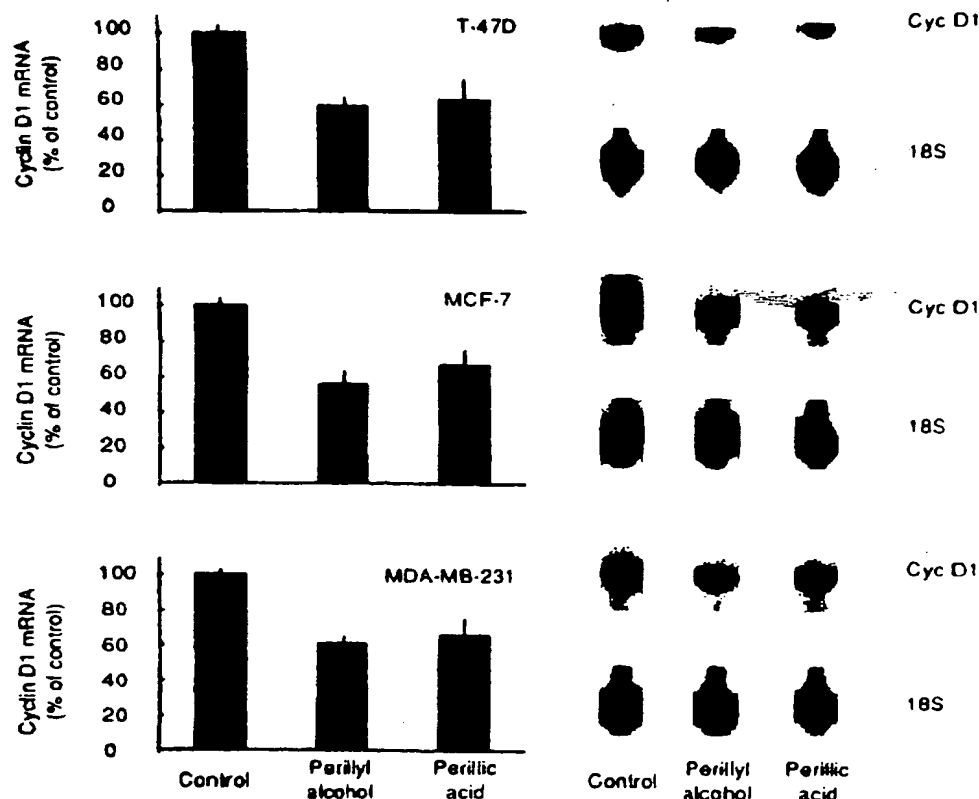
Because of the *in vivo* effects of monoterpenes on mammary tumors, it was of interest to study the direct effects of these compounds on the growth of human breast cancer cells.

In the present report we have clearly demonstrated that monoterpenes can inhibit the growth of human breast cancer cell lines in culture. Among the compounds tested, the most potent inhibitor of cell proliferation in the three cell lines studied was found to be perillyl alcohol, whereas limonene was the least effective inhibitor of cell proliferation. These results are in agreement with *in vivo* data showing that monoterpenes induce the regression of chemically induced rat mammary carcinomas and that perillyl alcohol is greater than five times more potent than limonene in regressing mammary tumors (7). The concentrations of monoterpenes required to inhibit cell proliferation (50% inhibition concentration = 0.1 mM for perillyl alcohol and 0.4 mM for perillic acid in the T-47D cell line) were similar to the serum levels of terpene metabolites in rats fed perillyl alcohol (7).

Because limonene and perillyl alcohol occur naturally in the (*R*) and (*S*) optically active forms, we tested the effects of both enantiomers on the growth of breast cancer cells. Results indicated that the effects of (*R*) enantiomers were not different from those of (*S*) enantiomers. A recent *in vitro* study in the PANC-1 pancreas carcinoma cell line has also reported that there is no enantiomeric specificity for antiproliferative effects of perillyl alcohol in these cells (11).

Several mechanisms of monoterpene action have been previously explored. First, it has been shown that monoterpenes induce changes in carcinogen-metabolizing hepatic enzymes in dimethylbenz[*a*]anthracene-induced mammary tumors in rats (18). On another hand, molecular studies have reported an increase in mannose 6-phosphate/insulin growth factor II receptor expression associated with an increase in transforming growth factor- $\beta$  expression in monoterpene-treated regressing mammary tumors (19,20). Finally, *in vitro* studies in NIH/3T3 fibroblasts and in M600B nontransformed mammary epithelial cells have shown that monoterpenes can inhibit the posttranslational isoprenylation of small G proteins such as growth-controlling Ras oncoproteins (21). However, a recent study has reported that limonene and perillyl alcohol are unlikely to inhibit cell growth by inhibiting Ras function in the PANC-1 pancreas carcinoma cell line (11).

To elucidate the effects of limonene-related monoterpenes on the growth of breast cancer cell lines, we tested their effects on T-47D cell cycle phase distribution. A pronounced decrease in the percentage of cells in the S phase and a corresponding increase in the proportion of cells in the G<sub>1</sub> phase were associated with antiproliferative action of perillyl alcohol and perillic acid. It has been reported that changes in breast cancer cell cycle distribution are often related to alterations of the expression of cell cycle-related genes and, especially, cyclin genes (17). Within the cyclin family, cyclin D1 plays a major role in cell cycle control



**Figure 5.** Modulation of cyclin D1 (Cyc D1) mRNA expression by monoterpenes in human breast cancer cells. T-47D, MCF-7, and MDA-MB-231 cells were exposed for 6 h to 0.5 mM perillyl alcohol, 1 mM perillic acid, or ethanol vehicle. Total RNA was extracted and analyzed by Northern blotting. Cyclin D1 hybridization signals were quantitated by electronic autoradiography and standardized to 18S rRNA signals. Values are expressed as percentage of cyclin D1 signal from control cells and are representative of 3 separate experiments.

and is considered a breast cancer oncogene (13). Several studies have reported that cyclin D1 gene expression can be inhibited by antiproliferative agents in breast cancer cell lines (17,22,23). We showed in this study that perillyl alcohol and perillic acid induced a reduction of cyclin D1 mRNA levels that preceded their effect on breast cancer cell cycle progression and cell growth.

In summary, we presented evidence that monoterpenes can affect the growth of breast cancer cells in culture. We showed that perillyl alcohol and perillic acid exerted a dose-dependent inhibitory effect on the proliferation of T-47D and MCF-7 hormone-dependent cells and MDA-MB-231 hormone-independent cells. Our data indicated a range in sensitivity among breast carcinoma cell lines. Of the three cell lines, T-47D was the most sensitive to antiproliferative effects of monoterpenes and MDA-MB-231 the least sensitive. Whether the difference in the sensitivity of the three cell lines to monoterpenes is linked to their hormone receptor status or other molecular genetic differences remains to be determined. The antiproliferative action of limonene-related monoterpenes was correlated with an accumulation of cells in the G<sub>1</sub> phase and a decrease in the proportion of cells in the S phase. This G<sub>1</sub>-S arrest was preceded by a decrease

in cyclin D1 mRNA levels, suggesting that the inhibition of cyclin D1 gene expression after monoterpene treatment may partially contribute to tumor cell growth suppression. Further studies, including detailed analysis of the effects of monoterpenes on the regulation of cell cycle-related genes, are necessary to clarify these findings. Such studies will improve understanding of the mechanisms of antiproliferative action of these new chemopreventive agents.

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# Systems for Identifying New Drugs Are Often Faulty

Screening potential anticancer drugs sounds easy. Just take a candidate drug, add it to a tumor type of choice, and then monitor whether the agent kills the cells or inhibits cancer growth. Too bad it hasn't been that simple. Even as investigators try to develop a new generation of more effective and less toxic anticancer drugs that directly target the gene changes propelling cells toward uncontrollable division (see p. 1036), they face a long-standing problem: sifting through potential anticancer agents to find ones promising enough to make human clinical trials worthwhile.

Indeed, since formal screening began in 1955, many thousands of drugs have shown activity in either cell or animal models, but only 39 that are used exclusively for chemotherapy, as opposed to supportive care, have won approval from the U.S. Food and Drug Administration. "The fundamental problem in drug discovery for cancer is that the model systems are not predictive at all," says Alan Oliff, executive director for cancer research at Merck Research Laboratories in West Point, Pennsylvania.

Pharmaceutical companies often test drug candidates in animals carrying transplanted human tumors, a model called a xenograft. But not only have very few of the drugs that showed anticancer activity in xenografts made it into the clinic, a recent study conducted at the National Cancer Institute (NCI) also suggests that the xenograft models miss effective drugs. The animals apparently do not handle the drugs exactly the way the human body does. And attempts to use human cells in culture don't seem to be faring any better, partly because cell culture provides no information about whether a drug will make it to the tumor sites.

The pressure is on to do better. So researchers are now trying to exploit recent discoveries about the subtle genetic and cellular changes that lead a cell toward cancer to create cultured cells or animal models that accurately reproduce these changes. "The real challenge for the 1990s is how to maximize our screening systems so that we are using the biological information that has accumulated," says Edward Sausville, associate director of the division of cancer treatment and diagnosis for the developmental therapeutics program at the NCI. "In short, we need to find faithful representations of carcinogenesis."

The first efforts to do so date back to the end of World War II, when hints began

emerging that some chemicals might have cancer-fighting effects. That evidence encouraged many chemists to explore the anticancer potential of similar agents shelved in their laboratories. And after commercial interests decided against helping the academics set up an efficient way to screen their chemicals, the NCI stepped in.

The institute started by pulling together mouse models of three tumors: a leukemia, which affects blood cells; a sarcoma, which arises in bone, muscle, or connective tissue; and a carcinoma, the most common type of cancer, which arises in epithelial cells and includes such major killers as breast, colon, and lung cancers. Initially, many of the agents tested in these models appeared to do well. However, most worked against blood cancers such as leukemia and lymphoma, as opposed to the more common solid tumors. And when tested in human cancer patients, most of these compounds failed to live up to their early promise.

Researchers blamed the failures on the fact that the drugs were being tested against mouse, not human, tumors, and beginning in 1975, NCI researchers came up with the xenograft models, in which investigators implant human tumors underneath the skin of mice with faulty immune systems. Because the animals can't reject the foreign tissue, the tumors usually grow unchecked, unless stopped by an effective drug. But the results of xenograft screening turned out to be not much better than those obtained with the original models, mainly because the xenograft tumors don't behave like naturally occurring tumors in humans—they don't spread to other tissues, for example. Thus, drugs tested in the xenografts appeared effective but worked poorly in humans. "We had basically discovered compounds that were good mouse drugs rather than good human drugs," says Sausville.

The xenograft models may also have

missed effective drugs. When Jacqueline Plowman's team at NCI tested 12 anticancer agents currently used in patients against 48 human cancer cell lines transplanted individually into mice, they found that 30 of the tumors did not show a significant response—defined as shrinking by at least 50%—to any of the drugs.

Researchers have not yet figured out why so many of the xenografts were insensitive to the drugs. But the NCI team says that the result means that drugs would have to be screened against six to 12 different xenografts to make sure that no active anticancer drugs were missed. That's an expensive proposition, as the average assay costs about \$1630 when performed by the government and \$2900 when done commercially. "I cannot get on my pulpit and say that the way we are doing this is the best way, because I don't think there is a good way to do it," says Sausville.

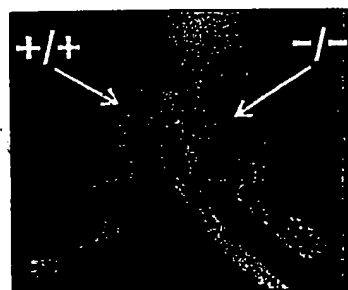
To create better models of cancer development in humans, investigators are now

drawing on the growing knowledge of human cancer-related gene mutations. They are genetically altering mice so that they carry the same kinds of changes—either abnormal activation of cancer-promoting oncogenes or loss of tumor-suppressor genes—that lead to cancer in humans. The hope is that the mice will develop tumors that behave the same way the human tumors do.

So far, the results from these mouse models have been mixed, however. One mutant mouse strain, for example, lacks a working APC gene, a tumor suppressor that leads to colon cancer when lost or inactivated. This mouse seems to do well at re-creating the early signs of colon cancer. But in the later stages of the disease, the type of mutations in the tumors

begin to diverge from those in human colon cancer, and the disease manifests itself differently as well. It spares the liver, for example, unlike the human cancer.

Other new mouse models have fared even worse. Take the one in which the retinoblastoma (RB) tumor-suppressor gene was knocked out. In humans, loss of RB leads to a cancer in the retina of the eye. But when the gene is inactivated in mice, the rodents get pituitary gland tumors. And BRCA1 knock-



**Not a matched pair.** In the clonogenic assay (top), tumor cells with (+/+) and without (-/-) the p21 gene responded similarly to radiation. But in mice, the p21- tumors often shrank, while those having the gene never did.

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—which are supposed to simulate human breast and ovarian cancer—don't get any tumors at all. "One might expect that these animals would also mimic human symptoms, not just the genetic mutations," says molecular biologist Tyler Jacks of the Massachusetts Institute of Technology. "In fact, that is usually the exception, not the rule."

Why gene knockouts in mice have effects so different from those of the corresponding mutations in humans is unclear. One possibility is that in mice, other genes can compensate for a missing gene, such as *BRCAL*. Another, says Jacks, is that "the genetic wiring for growth control in mice and humans is subtly different."

The limitations of animal models have spurred the NCI, among others, to test drug candidates in cultures of human cells. The institute now relies on a panel of 60 human tumor cell lines, including samples of all the major human malignancies. Drugs to be tested are fed to subsets of the panel, based on tumor cell type, and their cell-killing activity is monitored.

Over the last 7 years, the panel has been used to screen almost 63,000 compounds, and 5000 have exhibited tumor cell-killing activity. But that has created another dilemma, because so many compounds show antitumor cell activity in culture, and the cost of bringing them all to clinical trials—where most don't work anyway—would be daunting. As Sausville asks: "How do you prioritize so many compounds for clinical trials?" For that, the NCI uses a computer database to sift through past antitumor agents and look for only those compounds with novel mechanisms of action. Computer screening has whittled the number of promising agents down to about 1200, according to Sausville.

Those compounds are then tested in what is known as a hollow fiber model, in which tiny tubes filled with tumor cells are implanted into mice in a variety of sites. By monitoring the tumor cell-killing effects of drugs on the implants, researchers can test which drugs actually make it to the tumor sites when the drugs are administered in different ways: intravenously versus orally, for example. Sausville cautions, however, that it's still too early to tell how predictive these screens are, because only a few of the drugs tested have gone far enough to show efficacy in humans.

Both drug screeners and doctors also use another cell culture method, the so-called clonogenic assay, to sift through potential anticancer drugs. They grow cell lines or a patient's tumor cells in petri dishes or culture flasks and monitor the cells' responses to various anticancer treatments. But clono-

genic assays have their problems, too. Sometimes they don't work because the cells simply fail to divide in culture. And the results cannot tell a researcher how anticancer drugs will act in the body.

What's more, new results from Bert Vogelstein's group at Johns Hopkins University School of Medicine add another question mark about the assay's predictive ability. Todd Waldman, a postdoc in the Vogelstein laboratory, found that xenografts and clonogenic assays deliver very different messages about how cancer cells lacking a particular gene, *p21*, respond to DNA-crippling agents.

tumor cells, the entire tumor may shrink.

The finding indicates that the clonogenic assay can't always predict how a tumor will respond to a drug in an animal. Still, by linking the different responses in two models to the presence or absence of a specific gene system, the Waldman team's results help clarify why tumor cells might respond differently in culture and in animals. Indeed, the general idea that a tumor's drug sensitivity may be linked to the genetic mutations it carries has led others to try to use cells with comparable mutations to identify better chemotherapeutic agents.

Leland Hartwell, Stephen Friend, and their colleagues at the Fred Hutchinson Cancer Research Center in Seattle are pioneering one such effort. They are building on previous work in which Hartwell's team discovered a series of yeast genes, called checkpoint genes, that normally stop cells from progressing through the cell cycle and dividing if they have abnormalities such as unrepaired DNA damage. Because mutations in checkpoint and other cell cycle-related genes have been linked to human cancers, looking for

drugs that restore normal growth control in mutated yeast might be one way to find new cancer therapies (see Article on p. 1064).

The NCI is taking a similar tack. They are looking to see if they can reclassify the cells in their panel, which was set up based on tissue type—breast cancer versus colon cancer, for example—according to the types of genetic defects the cells carry. To enable drugs that counteract specific defects to be prescribed most effectively, researchers are also developing technologies for analyzing the gene defects in each patient's tumors. That way, if drugs that correct specific defects can be identified, they could then be matched to each individual's tumor cell makeup. "This would be so valuable," says Homer Pearce, vice president of cancer research and clinical investigation at Eli Lilly and Co. in Indianapolis. "It would help to identify patients that have the greatest chance of benefiting from therapy, while minimizing the number that would be exposed to a treatment that would not work."

Indeed, Merck's Oliff says, "the future of cancer drug screening is turning almost exclusively toward defining molecular targets." If the approach works, drug developers would finally have an easy way to identify promising cancer drugs, and cancer patients might have an array of new treatments.

—Trisha Gura

#### TESTING THE XENOGRAFT ASSAY

Type of Cancer	Cell Lines Tested	% of Tumors Responding to Drugs	
		Minimal Response (< 40% shrinkage)	Significant Response (> 50% shrinkage)
Colon	9	31%	5%
Brain	1	0%	20%
Lung, non-small cell	7	49%	8%
Lung, small cell	3	63%	9%
Breast	6	51%	19%
Pancreatic	1	0%	1%
Ovarian	3	35%	0
Prostate	2	0%	0
Renal	5	43%	0

Radiation, like many of the drugs used to treat cancer, works by damaging the cells' DNA. This either brings cell replication to a halt or triggers a process known as apoptosis in which the cells essentially commit suicide. Waldman wanted to see how *p21*, one of the genes involved in sensing the DNA damage and halting cell replication, influences that response to radiation.

In the mouse xenograft assay, Waldman and his colleagues found that the radiation cured 40% of the tumors composed of cells lacking *p21*, while tumors made of cells carrying the gene were never cured. But this difference was not apparent in the clonogenic assay, where the radiation appeared to thwart the growth of both dispersed tumor cell types. "We showed this gross difference in sensitivity in real tumors in mice and in the clonogenic assay," Waldman says.

He suggests that the different responses in the two systems have to do with the fact that a subset of *p21* mutants die in response to radiation, while cells with the normal gene merely arrest cell division. Either way, the dispersed tumor cells in the clonogenic assay will fail to grow. However, in the xenograft tumors, which consist of many cells in a solid mass, the arrested, but nonetheless living, *p21*<sup>+</sup> tumor cells may release substances that encourage the growth of any nearby tumor cells that escaped the effects of the radiation. But tumor cells lacking the *p21* gene die, and because dead cells cannot "feed" neighboring

Trisha Gura is a writer in Cleveland, Ohio.

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 PB Fischer  
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 CC 1-7 (Pharmacology)  
 AB Eugenol is an active principle and responsible for several pharmacol. activities of clove oil. The authors studied the effects of **eugenol** on human **platelet** **aggregation**, arachidonic acid (AA) and **platelet**-activating factor (PAF) metab. and in vivo effects on AA and PAF-induced shock in rabbits. **Eugenol** strongly inhibited PAF-induced **platelet** **aggregation** with lesser effect against AA and collagen. The IC50 values were against AA: 31 .mu.M; collagen: 64 and PAF 7 .mu.M, resp. In addn., eugenol stimulated PAF-acetylhydrolase activity suggesting that inhibition of PAF could be due to its inactivation to lyso-PAF. Pretreatment of rabbits with eugenol (50-100 mg/kg) prevented the lethal effects of i.v. PAF (11 .mu.g/kg) or AA (2 mg/kg) in a dose-dependent fashion. The protective effects of eugenol in the rabbits, however, were more pronounced against PAF-induced mortality (100% protection). In addn., eugenol also inhibited AA metab. via cyclooxygenase and lipoxygenase pathways in human platelets. Both the prodn. of thromboxane-A2 and 12-hydroxy-eicosatetraenoic acid was inhibited by eugenol in a concn.-related manner (30-120 .mu.M). In vivo, eugenol (50-100 mg/kg; i.p.) inhibited carrageenan-induced rat paw edema. In this test, eugenol was 5 times more potent than aspirin. These results provide evidence that eugenol acts as a dual antagonist of AA and PAF.  
 ST eugenol platelet activating factor arachidonate inflammation  
 IT Blood platelet  
 Inflammation inhibitors  
 (eugenol as dual inhibitor of platelet-activating factor and arachidonic acid metab.)  
 IT Collagens, biological studies  
 RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)  
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 RL: ADV (Adverse effect, including toxicity); BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)  
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 RL: ADV (Adverse effect, including toxicity); BSU (Biological study, unclassified); MFM (Metabolic formation); BIOL (Biological study); FORM (Formation, nonpreparative)  
 (eugenol as dual inhibitor of platelet-activating factor and arachidonic acid metab.)  
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 RL: BSU (Biological study, unclassified); MFM (Metabolic formation); BIOL (Biological study); FORM (Formation, nonpreparative)  
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Examiner

Patricia Patten

Group Art Unit

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## Minireview

# Interactions between the Epidermal Growth Factor Receptor and Type I Protein Kinase A: Biological Significance and Therapeutic Implications<sup>1</sup>

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## Abstract

Peptide growth factors regulate normal cellular proliferation and differentiation through autocrine and paracrine pathways and are involved in cancer development and progression. Among the endogenous growth factors, the epidermal growth factor (EGF)-related proteins play an important role in the pathogenesis of human cancer. In fact, overexpression of EGF-related growth factors such as transforming growth factor  $\alpha$  and amphiregulin and/or their specific receptor, the EGF receptor (EGFR), has been detected in several types of human cancers, including breast, lung, and colorectal cancers. Therefore, the blockade of EGFR activation by using anti-EGFR monoclonal antibodies (MAbs) has been proposed as a potential anticancer therapy.

The cAMP-dependent protein kinase (PKA) is an intracellular enzyme with serine-threonine kinase activity that plays a key role in cell growth and differentiation. Two PKA isoforms with identical catalytic (C) subunits but different cAMP-binding regulatory (R) subunits (defined as RI in PKAI and RII in PKAII) have been identified. Predominant expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKAI are detected steadily in tumor cells and transiently in normal cells exposed to mitogenic stimuli. Overexpression of PKAI has been correlated recently with poor prognosis in breast cancer patients. Inhibition of PKAI expression and function by specific pharmacological agents such as the selective cAMP analogue 8-chloro-cAMP (8-Cl-cAMP) induces growth inhibition in various human cancer cell lines *in vitro* and *in vivo*.

We have provided experimental evidence of a functional cross-talk between ligand-induced EGFR activation and PKAI expression and function. In fact, PKAI is overexpressed and activated following transforming growth fac-

tor  $\alpha$ -induced transformation in several rodent and human cell line models. Furthermore, PKAI is involved in the intracellular mitogenic signaling following ligand-induced EGFR activation. We have shown that an interaction between EGFR and PKAI occurs through direct binding of the RI subunit to the Grb2 adaptor protein. In this respect, PKAI seems to function downstream of the EGFR, and experimental evidence suggests that PKAI is acting upstream of the mitogen-activated protein kinase pathway.

We have also demonstrated that the functional interaction between the EGFR and the PKAI pathways could have potential therapeutic implications. In fact, the combined interference with both EGFR and PKAI with specific pharmacological agents, such as anti-EGFR blocking MAbs and cAMP analogues, has a cooperative antiproliferative effect on human cancer cell lines *in vitro* and *in vivo*. The antitumor activity of this combination could be explored in a clinical setting because both the 8-Cl-cAMP analogue and the anti-EGFR blocking MAb C225 have entered human clinical trial evaluation.

Finally, both MAb C225 and 8-Cl-cAMP are specific inhibitors of intracellular mitogenic signalling that have different mechanisms of action compared with conventional cytotoxic drugs. In this respect, a cooperative growth-inhibitory effect in combination with several chemotherapeutic agents in a large series of human cancer cell lines *in vitro* and *in vivo* has been demonstrated for anti-EGFR blocking MAbs or for 8-Cl-cAMP. Therefore, the combination of MAb C225 and 8-Cl-cAMP following chemotherapy could be investigated in cancer patients.

## Introduction

Growth factors regulate normal cellular proliferation and differentiation and are important in initiating and maintaining neoplastic transformation (1). Cancer cells generally exhibit a decreased requirement for exogenous growth factors as compared with normal cells (2). The relaxation in growth factor dependency is due in part to the ability of tumor cells to synthesize growth factors that can regulate their proliferation through autocrine and paracrine mechanisms by activating specific cell membrane receptors (2). Among the endogenously produced peptide growth factors, TGF- $\alpha$ ,<sup>3</sup> AR, and CRIPTO are

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<sup>3</sup> The abbreviations used are: TGF, transforming growth factor; AR, amphiregulin; EGF, epidermal growth factor; EGFR, EGF receptor; Grb2, growth factor receptor binding protein 2; SH, Src homology; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase; 8-Cl-cAMP, 8-chloro-cAMP; C, catalytic subunit; R, regulatory subunit; TSH, thyroid-stimulating hormone; MAb, monoclonal antibody; MBO, mixed backbone oligonucleotide.



EGF-related proteins that play an important role in the pathogenesis of several human epithelial cancers (3). TGF- $\alpha$ , AR, and CRIPTO are expressed by the majority of human primary and metastatic breast and colorectal cancers (4-5). Suppression of synthesis of these growth factors by pharmacological tools such as a specific RNA or DNA antisense approach inhibits human colon and breast cancer cell growth (6-9). Both TGF- $\alpha$  and AR bind to and activate the EGFR. Enhanced expression of EGFR has been detected in the majority of glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas (3). Overexpression of EGFR has been associated with a poor prognosis in several human tumor types, including breast cancer (3). The EGFR is a  $M_r$  170,000 transmembrane glycoprotein with an external binding domain and an intracellular tyrosine kinase domain. Following ligand binding, the EGFR is autophosphorylated on several tyrosine residues in the intracellular domain and dimerizes, creating a series of high-affinity binding sites for various adaptor molecules that are involved in transmitting the mitogenic signaling to the *ras*/MAPK signal transduction pathway (10). In this respect, Grb2 is an adaptor molecule composed of one SH2 domain, which binds to phosphorylated tyrosines on tyrosine kinase receptors, and of two SH3 domains, which bind to proline-rich sequences of signaling proteins such as SOS (11). Grb2 allows the coupling of the activated EGFR to *ras*, phosphatidylinositol kinase, or phospholipase C $\gamma$  pathways (11-15). The specific cell membrane receptor for CRIPTO has not yet been identified, although the addition of recombinant CRIPTO protein to human mammary epithelial cells induces the intracellular signaling cascade that leads to MAPK activation (16).

The PKA is an intracellular enzyme with serine-threonine kinase activity that plays a key role in cell growth and differentiation. cAMP acts in mammalian cells by binding to either of two distinct isoforms of PKA, defined PKAI and PKAII. PKAI and PKAII share identical catalytic (C) subunits, but differ in the regulatory (R) subunits (termed RI in PKAI and RII in PKAII, respectively; Ref. 17). The PKA holoenzyme is a tetramer formed of two identical R subunits and two C subunits (17). Upon cAMP binding to the R subunits, the active C subunit is released (17). The synthesis of RI and RII and the relative abundance of PKAI and PKAII isoforms are differentially regulated during differentiation, cell growth, and neoplastic transformation (18). Predominant expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKAI are detected steadily in tumor cells and transiently in normal cells exposed to mitogenic stimuli (19). In this respect, PKAI and its regulatory subunit RI $\alpha$  are generally overexpressed in human cancer cell lines and primary tumors and are induced following transformation by certain oncogenes, such as *ras* (18-19). Overexpression of RI $\alpha$  and PKAI has been correlated recently with poor prognosis in breast cancer patients (20). RI $\alpha$  has also been identified in hepatoma  $\times$  fibroblast cell hybrids as the tissue extingisher of differentiation (TSB1), an inhibitor of the expression of several genes related to cell differentiation (21). In contrast, constitutive overexpression of PKAII, following infection with a recombinant retrovirus containing the human *R/1B* gene, induces growth inhibition of human cancer cells, and reverts the transformed phenotype of *ras*-transformed mouse fibroblasts (22-23). Collectively, these data, along with the distinct subcellular location

and the differential sensitivity to cAMP concentration for enzyme activation (17-18), suggest that PKAI and PKAII have different functions in the control of cell growth and differentiation.

In recent years, experimental evidence has been provided on a functional link between neoplastic transformation involving the TGF- $\alpha$ -EGFR autocrine pathway and PKAI expression and activity. In this review, we will discuss the biological relevance of the interactions between the activated EGFR and PKAI and we will present data suggesting that the blockade with specific biological agents of the PKAI serine-threonine kinase-dependent pathway and of the EGFR tyrosine kinase-dependent pathway is a potentially useful novel approach in cancer therapy.

### The EGFR-PKAI Connection

**PKAI Expression Is Linked to TGF- $\alpha$ - and *ras*-dependent Transformation.** Several studies have shown an increased expression of RI $\alpha$  and PKAI following transformation by TGF- $\alpha$  or *ras*. On the other hand, an early inhibition of TGF- $\alpha$  and/or *ras* expression is observed after treatment with selective inhibitors of PKAI, such as 8-Cl-cAMP. These studies have suggested a functional involvement of PKAI in the mitogenic signals transmitted through the EGFR and/or p21/*ras* pathways. For example, in NRK rat fibroblasts, TGF- $\alpha$ - and *ras*-dependent transformation causes an early induction of RI $\alpha$  and PKAI expression and a parallel reduction of PKAII (24). The selective inhibition of PKAI by 8-Cl-cAMP is followed by inhibition of TGF- $\alpha$  and p21/*ras* expression, by increased PKAII expression, and by cell growth arrest (24). In NOG-8 mouse mammary epithelial cells, stable overexpression of TGF- $\alpha$ , following transfection with a recombinant plasmid containing the human TGF- $\alpha$  cDNA, determines neoplastic transformation (25). This event is associated with a fall in R1B mRNA expression and a parallel rise of RI $\alpha$  mRNA expression, without major changes in C $\alpha$  expression (26). Therefore, PKAI becomes the predominant PKA isoform in TGF- $\alpha$ -transformed NOG-8 cells. Treatment of these cells with the specific PKAI inhibitor 8-Cl-cAMP down-regulates PKAI, induces PKAII, and inhibits TGF- $\alpha$  production, restoring the pattern of expression of non-transformed parental cells (26). Similarly to the NOG-8 mouse model, in MCF-10A normal human mammary epithelial cells, overexpression of TGF- $\alpha$  or of an activated *ras* gene causes neoplastic transformation (27). These events are associated with an increase of PKAI expression and a parallel reduction of PKAII. Down-regulation of PKAI by different pharmacological approaches, such as cAMP analogues or antisense oligonucleotides targeted against RI $\alpha$ , inhibits TGF- $\alpha$  expression and induces growth inhibition in MCF-10A cells transformed by either TGF- $\alpha$  or *ras* (28). Further experimental evidence of a functional cross-talk between TGF- $\alpha$ -EGFR-mediated cell transformation and PKAI expression and function has been provided recently in MDA-MB468 human breast carcinoma cells. In MDA-MB468 cells, a TGF- $\alpha$ -EGFR autocrine growth stimulatory pathway is involved in the control of cell growth and transformation (29). The constitutive inhibition of EGFR expression by transfection of MDA-MB468 cells with an antisense

EGFR plasmid vector is accompanied by the selective down-regulation of PKAI expression (30).

**PKAI Is Induced by EGFR Activation in Normal Cells.** Different studies have disclosed a link between PKA activation and inhibition of EGFR- and/or *ras*-dependent activation of MAPK (31–33), but whether a specific PKA isoform is responsible for this effect has not yet been elucidated. On the other hand, it has been shown that an activated *ras* oncogene inhibits the function of PKA by interfering with the nuclear location of the  $\alpha$  catalytic subunit, conceivably following release from PKAII (34). Recently, it has been demonstrated that treatment with the cAMP analogue 8-bromo-cAMP or with cAMP-specific phosphodiesterase inhibitors determines inhibition of smooth muscle cell proliferation in rat carotid arteries following injury (35). Because smooth muscle cell proliferation has been shown to be dependent on *ras/raf-1*/MAPK signaling in this model (36), it seems likely that the growth-inhibitory effect is due to the interaction of PKA and *ras*-mediated signals. PKAI has been causally linked to positive regulation of mitogenic signals through the EGFR and *ras* pathways (24, 26, 28). We have shown that PKAI expression is induced in nontransformed MCF-10A human mammary epithelial cells following treatment with EGF or TGF- $\alpha$ , and it is functionally involved in S-phase entry (37). MCF-10A cells possess approximately 250,000 EGFR sites/cell and depend on the presence of EGF or TGF- $\alpha$  in the culture medium for optimal cell growth, because their withdrawal determines growth arrest in the G<sub>0</sub>-G<sub>1</sub> phases of the cell cycle. The addition of complete medium containing EGF to quiescent MCF-10A cells induces R1 $\alpha$  expression 6–9 h before cells enter S phase (37). Selective down-regulation of PKAI expression by pretreatment with an anti-R1 $\alpha$  antisense oligonucleotide blocks S-phase entry of MCF-10A cells following EGF addition, suggesting a role for PKAI in the EGFR-triggered G<sub>1</sub>-to-S transition (37). Furthermore, retroviral vector-mediated R1 $\alpha$  overexpression enables MCF-10A cells to grow in serum-free medium, bypassing EGF or TGF- $\alpha$  requirement and conferring a phenotype similar to MCF-10A cells transformed by either the TGF- $\alpha$  or the *ras* genes (27, 37). Taken together, these data suggest that PKAI mediates the mitogenic signaling by growth factors of the EGF family in human mammary epithelial cells.

It is not yet clear whether PKAI activation is involved only in the downstream propagation of the EGFR-induced mitogenic signaling or whether PKAI is part of the signal transduction cascade induced by other growth factors. In this respect, an early involvement of PKAI following other mitogenic stimuli in different cell types has been reported. In normal human T lymphocytes, CD3 stimulation or phytohemagglutinin addition causes specific PKAI induction and activation within 5–10 min (38–39). In FRTL-5 rat thyroid cells, which depend on TSH for cell proliferation and thyroglobulin synthesis, a rapid induction of R1 $\alpha$  mRNA occurs within 30 min after TSH addition with an increase in PKAI that anticipates cell entry into S phase (40). Conversely, inhibition of PKAI synthesis by an anti-R1 $\alpha$  antisense oligonucleotide abrogates the TSH-induced mitogenic effect (40). Interestingly, a tumor-specific transforming sequence derived by the fusion of the *ret* tyrosine kinase receptor and the R1 $\alpha$  genes has been isolated in two human papillary thyroid carcinomas (41).

**Direct Interaction of PKAI with the Activated EGFR through Grb2.** It is possible that the different biological effects of the two PKA isoforms is due in part to their intracellular localization, which could allow interactions with potentially different adaptor molecules and/or substrates. The subcellular distribution of the PKA isoforms depends also on the interaction with a specific class of anchoring proteins (AKAPs), which may contribute to their functional role (42). PKAII has been found in association with the plasma membrane, the cytoskeleton, the secretory granules, and the nucleus (19, 43–45). PKAI is broadly distributed in the cytoplasm (46) and may also translocate to the cell membrane. In fact, in human T lymphocytes, PKAI is found in the inner face of the cell membrane, where it is associated with the T-cell receptor-CD3 complex after T-cell activation (39, 47). In EGF-stimulated MCF-10A cells, a cell membrane translocation of PKAI anticipates cell entry into S phase (37, 48).

Furthermore, we have shown recently that in MCF-10A both the R1 $\alpha$  and  $\alpha$  subunits, but not the RII $\beta$  subunit, coprecipitate with the ligand-activated EGFR and that they are present in the EGFR macromolecular signaling complex as an activatable PKAI holoenzyme (49). Whole-cell immunofluorescence studies have shown that R1 $\alpha$  staining is superimposable to that of EGFR,<sup>4</sup> which, following ligand activation, translocates from the cell membrane to the cytoplasm by endocytosis (15).

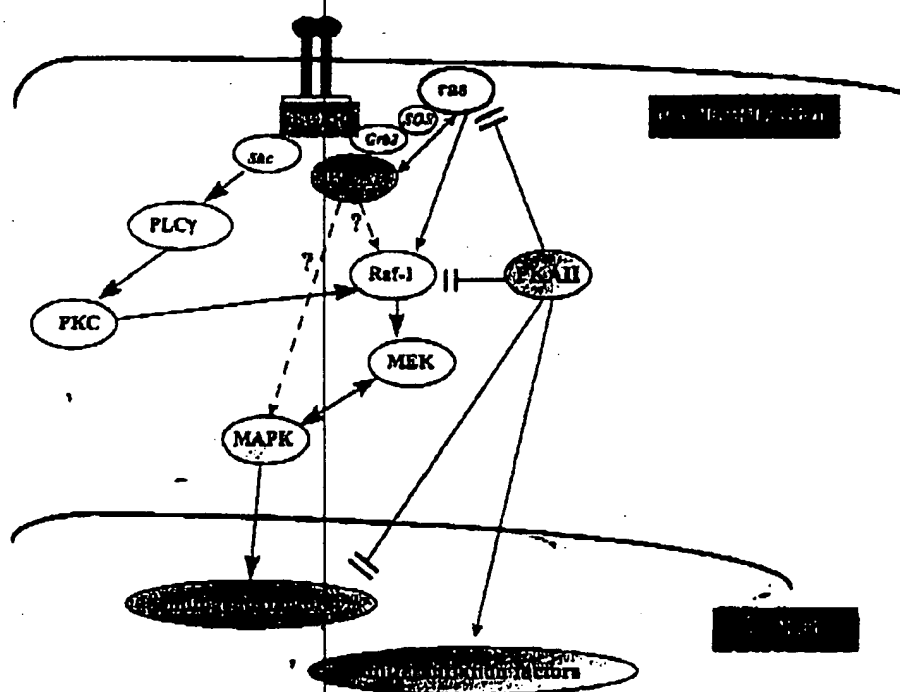
PKAI provides a relevant contribution to the propagation of EGFR-activated mitogenic intracellular signaling. In fact, overexpression of PKAI in MCF-10A R1 $\alpha$  cells determines a constitutive activation of MAPK, mimicking the effect of EGF addition to quiescent MCF-10A cells (49). In contrast, inhibition of PKAI-mediated signaling by a R1 $\alpha$  antisense oligonucleotide or by 8-Cl-cAMP significantly reduces MAPK activation in EGF-stimulated MCF-10A cells (49).

R1 $\alpha$  contains a stretch of uncharged amino acids and a NH<sub>2</sub>-terminal proline-rich sequence (47), which may potentially bind to SH3 domains (11). We have found that PKAI interacts with the EGFR through R1 $\alpha$  binding to either NH<sub>2</sub>- or COOH-terminal SH3 domains of Grb2 (49). R1 $\alpha$  is associated with Grb2 independently from EGFR activation, suggesting that R1 $\alpha$  and Grb2 may form a complex before ligand activation of EGFR and recruitment of Grb2 to autophosphorylated tyrosine residue(s) (49). Because an activatable PKAI holoenzyme is present at the EGFR site following ligand-dependent activation, PKAI may interact with specific substrates involved in the EGFR-dependent signaling cascade. However, it is not yet defined whether PKAI is involved in a specific signaling pathway or participate to the integration of multiple growth factor-induced signals. The identification of PKAI-specific substrates will be an important step to elucidate the role of this PKA isoform in the transduction of mitogenic signals (Fig. 1).

#### Therapeutic Implications

**Inhibition of EGFR.** Because experimental and clinical studies have provided evidence for a TGF- $\alpha$ -mediated autocrine

<sup>4</sup> Unpublished results.



**Fig. 1** PKA isoforms in the control of mitogenic signaling. PKAI participates to the EGFR-dependent mitogenic signaling through the binding of the R1 $\alpha$  subunit with the SH3 domain of Grb2 adaptor protein recruited at the activated tyrosine receptor site (49). EGFR signaling is not exclusively mediated by PKAI because other pathways are involved, including the phospholipase C $\gamma$ /PKC transducing molecules. Inhibition of PKAI markedly reduces MAPK activation (49), but it is unknown whether PKAI directly signals MAPK and raf-1. On the other hand, PKAII is able to inhibit PKAI, ras, raf-1, and, indirectly, MAPK function and expression (18, 31–34). Moreover, PKAII has an inhibitory effect on several nuclear mitogenic factors and a transactivating effect on factors involved with cell differentiation (18, 34). PLC $\gamma$ , phospholipase C $\gamma$ ; MEK, MAPK/extracellular signal-regulated kinase.

growth stimulatory pathway in a variety of human cancers, the blockade of the TGF- $\alpha$ -EGFR autocrine pathway by using anti-EGFR blocking MABs, recombinant proteins containing TGF- $\alpha$  or EGF fused to toxins, or EGFR-specific tyrosine-kinase inhibitors has been proposed as a potential therapeutic modality (50–57). In this respect, several blocking anti-EGFR MABs that inhibit the *in vitro* and *in vivo* growth of human cancer cell lines that express TGF- $\alpha$  and EGFR have been generated (58–61). Among these, MAB 528 and MAB 225 are two mouse MABs that have been extensively characterized for their biological and preclinical properties and represent the first series of anti-EGFR blocking agents that have entered clinical evaluation in cancer patients (50–51, 58–59). MAB 528 and MAB 225 bind to the EGFR with affinity similar to EGF and TGF- $\alpha$ , compete with these ligands for receptor binding, and block EGF- or TGF- $\alpha$ -induced activation of EGFR tyrosine kinase (58–59). In addition, it has been shown that the combined treatment of mice bearing well-established human tumor xenografts with MAB 528 or with MAB 225 and with cytotoxic drugs, such as doxorubicin or cisplatin, significantly increases the antitumor activity of these drugs (62–63). To avoid human anti-mouse antibody production that can interfere with the therapeutic efficacy of repeated administrations of mouse MABs in humans, a chimeric human-mouse MAB 225 (MAB C225), that contains the human IgG1 constant region, has been developed recently and purified for clinical use and is in early clinical trials in patients with advanced cancer (64–65).

**Inhibition of PKAI** The potential usage of cAMP analogues for the therapy of cancer has been widely discussed in the past two decades. However, the lack of selectivity and the high doses required for the available cAMP analogues have been a

major obstacle to the development of this approach into feasible clinical trials. A renewed interest has been fostered by the discovery of a new class of site-selective cAMP analogues, which are able to modulate the activity of the PKA at micromolar concentrations (18). 8-Cl-cAMP, the most potent site-selective cAMP analogue, can discriminate between the two cAMP binding sites on RI and RII and is able to down-regulate RI $\alpha$  by facilitating the degradation of the protein, while up-regulating RII expression at the transcriptional level (18, 66). We have shown that down-regulation of RI $\alpha$  by 8-Cl-cAMP is associated with growth inhibition (with IC<sub>50</sub>s ranging between 0.01 and 10  $\mu$ M) and differentiation in a wide variety of human cancer cell lines *in vitro* and *in vivo* (66–68). These effects are accompanied by an increased RII:RI ratio and by inhibition *in vitro* and *in vivo* of different oncogenes and growth factor expression, including *ras*, *myc*, *erbB2*, TGF- $\alpha$ , basic fibroblast growth factor, and vascular endothelial growth factor (24, 26, 28, 66–69). The inhibition in the expression of these genes induced by 8-Cl-cAMP treatment is time and dose dependent and occurs at the mRNA level (26, 69). It has been shown recently that 8-Cl-cAMP is able to revert multidrug resistance in a variety of multidrug resistance cancer cell lines, restoring the sensitivity to cytotoxic drugs (70). Although it is still debated whether metabolites such as 8-Cl-adenosine may contribute to the 8-Cl-cAMP effect (19), 8-Cl-cAMP is presently under clinical investigation in Phase II trials, because we have shown in a Phase I clinical trial that 8-Cl-cAMP can be safely administered to cancer patients at doses that achieve plasma concentrations within the potential therapeutic range for growth inhibition (71).

A more direct approach to inhibit the synthesis and function of PKAI has been developed by the use of phosphorothio-

site-modified antisense oligonucleotides targeted against the 5' coding sequence of the human *R1α* mRNA. Treatment with these antisense oligonucleotides suppressed *R1α* production and determined inhibition of *in vitro* proliferation in various human cancer cell lines and *in vivo* growth of LS-174T human colon cancer xenografts (72-74). Although phosphorothioate-modified antisense oligonucleotides have shown promising results as a first generation of oligonucleotides, a series of novel MBOs targeted against *R1α* has been generated to further improve their therapeutic potential. MBOs have appropriately placed segments of phosphorothioate oligonucleotides and segments of modified oligodeoxy- or oligoribonucleotides, such as methylphosphonate linkages (75). The anti-*R1α* second generation antisense oligonucleotides have a significant antiproliferative effect *in vitro* (with  $IC_{50}$ s ranging between 0.01 and 1  $\mu$ M) and *in vivo* in a number of human cancer cell lines.<sup>4</sup> Because MBOs have shown a significant reduction of side effects and a better pharmacokinetic profile *in vivo* as compared with phosphorothioate oligonucleotides (75), they are entering clinical evaluation in cancer patients.

**Combined Blockade of EGFR and PKAI.** The large body of experimental evidence suggesting a functional link between neoplastic transformation involving TGF- $\alpha$ -induced EGFR activation and PKAI has prompted studies to evaluate whether the double blockade of EGFR and PKAI may have an antiproliferative effect in human cancer cells and may improve the antitumor activity of either blockade alone. In a first series of experiments, we have evaluated the growth-inhibitory effects of the combined treatment with the anti-EGFR MAb 528 and 8-Cl-cAMP on two human colon cancer cell lines (GEO and CBS) and on a human breast cancer cell line (MDA-MB468; Ref. 76). The combination treatment with these two agents had a more than additive growth-inhibitory effect on all three cancer cell lines that secrete TGF- $\alpha$  and express functional EGFRs (76). A 3- to 5-fold reduction in the 8-Cl-cAMP  $IC_{50}$  was observed when the tumor cells were exposed to low noninhibitory doses of MAb 528 in combination with 8-Cl-cAMP. Furthermore, treatment with higher concentrations of MAb 528 and 8-Cl-cAMP determined a similar degree of cooperative growth inhibition. We have next demonstrated that the combination of the humanized chimeric anti-EGFR MAb C225 and 8-Cl-cAMP is a highly effective anticancer treatment regimen *in vivo* using human GEO colon carcinoma xenografts as a model (77). The combined blockade of EGFR and of PKAI produced an antitumor effect that is not simply additive. Treatment with low doses of MAb C225 and 8-Cl-cAMP for 5 weeks resulted in a long-term suppression of GEO tumor growth, because tumors resume their growth only after ~8 weeks from cessation of the treatment (77). This effect was accompanied by a statistically significant benefit in animal survival in the group treated with both agents as compared with the groups treated with a single agent. The anticancer effect of the MAb C225 plus 8-Cl-cAMP combination was also accompanied by the suppression in tumor cell production of proteins that function as autocrine growth factors, such as TGF- $\alpha$ , AR, and CRPTO, or as paracrine angiogenic growth factors, such as vascular endothelial growth factor and basic fibroblast growth factor (77). In addition, a strong inhibition in tumor-induced host neoangiogenesis was observed. The suppression of synthesis of endogenous growth factors has also

potential therapeutic relevance. This effect could lead to tumor quiescence in terms of proliferation and neoangiogenic stimulation. Therefore, long-term treatment with anticancer agents that affect intracellular signaling, such as anti-EGFR MABs and 8-Cl-cAMP, may obtain a control of cancer cell growth and spreading with no toxicity. In fact, MAb C225 and 8-Cl-cAMP treatments were well tolerated by the animals because no signs of toxicity were observed in any treatment group.

We are presently testing whether a similar cooperative antitumor effect could be also obtained by a combination of anti-*R1α* MBOs and anti-EGFR MABs. Preliminary experiments in human breast and renal carcinoma cell lines have shown a supraditive growth-inhibitory effect with this approach.<sup>4</sup>

## Conclusions

PKAI expression and activation is involved in the intracellular mitogenic signaling following EGFR activation. The interaction between EGFR and PKAI occurs through direct binding of the *R1α* subunit to the Grb2 adaptor. Therefore, PKAI seems to function downstream to the EGFR, and experimental evidence suggests that PKAI is acting upstream to the MAPK pathway.

The combined interference with EGFR and PKAI with specific pharmacological agents, such as anti-EGFR blocking MABs and cAMP analogues, has a cooperative antiproliferative effect on human cancer cell lines *in vitro* and *in vivo*. The antitumor activity of this combination could be explored in a clinical setting because both 8-Cl-cAMP and MAb C225 have entered human clinical trial evaluation.

Finally, both anti-EGFR MABs and 8-Cl-cAMP are inhibitors of intracellular mitogenic signaling with different mechanisms of action compared with cytotoxic agents. In this respect, a cooperative growth-inhibitory effect in combination with several conventional cytotoxic drugs in a large series of human cancer cell lines *in vitro* and *in vivo* has been demonstrated for anti-EGFR blocking MABs or for 8-Cl-cAMP (62-63, 78). Therefore, the combination of MAb C225 and 8-Cl-cAMP following chemotherapy could be investigated in cancer patients.

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## Suppressed mutagenicity of benzo[*a*]pyrene by the liver S9 fraction and microsomes from eugenol-treated rats

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### Summary

The mutagenicity of benzo[*a*]pyrene (B[*a*]P) in the Ames test using liver S9 fraction prepared from rats pretreated with eugenol (4-allyl-2-methoxyphenol) was suppressed to a lower level than that obtained using liver S9 from untreated rats. There was a reverse correlation between the mutagenicity of B[*a*]P and the dose of eugenol administered to the animals. Similarly suppressed mutagenicity was observed when liver microsomes, instead of the S9 fraction, were used in the Ames test. The mutagenic activity of B[*a*]P and arylhydrocarbon hydroxylase (AHH) could not be inhibited by the direct addition of eugenol into the assay mixtures. In eugenol-treated microsomes, cytochrome P-450 content, AHH activity and total B[*a*]P hydroxylase activity were decreased to 81, 29 and 48% of the control values, respectively. The mutagenicity of B[*a*]P catalyzed by microsomes from rats fed ad libitum on a diet containing 5% eugenol in the Ames test was significantly decreased. AHH activity and total B[*a*]P hydroxylase activity were also decreased in these liver microsomes. These results indicate that the activation of B[*a*]P in rat liver by cytochrome P-450, which metabolizes B[*a*]P to ultimate mutagens or carcinogens, is reduced by the administration of eugenol.

Some edible plants and a variety of substances in them have been reported to inhibit carcinogenesis or mutagenesis [1]. Recently, it was reported that ellagic acid and some plant phenols inhibited the mutagenicity of ultimate carcinogenic metabolites of B[*a*]P [12]. Several chalcone derivatives [10] and aqueous and acetone extractions of some common vegetables [5] were found to reduce the revertant increase of *Salmonella typhimurium* TA100 by B[*a*]P. These antimutagens inhibit the mutagenic activity of B[*a*]P in the *Salmonella*

mutagenicity test during incubation with B[*a*]P and S9 mix in vitro.

Eugenol, a principal constituent of clove oil, is a widely used flavor additive and chemical intermediate. Recently, it has been reported [17] that no evidence of carcinogenicity due to eugenol was observed in male or female rats, and eugenol did not inhibit or induce a mutagenic response observed in mutagenesis studies using *Salmonella typhimurium*. In previous papers [14-16], we reported the enhancement of glucuronidation and the induction of liver microsomal UDP-glucuronyltransferase by oral administration of eugenol to rats. The present paper reports that the mutagenicity of B[*a*]P in the Ames test using liver S9 or microsomes prepared from rats administered

**Abbreviations:** AHH, arylhydrocarbon hydroxylase; B[*a*]P, benzo[*a*]pyrene; BHA, (2)-tert-butyl-4-hydroxyanisole; 3-MC, 3-methylcholanthrene; PB, phenobarbital; PCBs, polychlorinated biphenyls.



eugenol is decreased in comparison to B[a]P mutagenicity using untreated rat liver S9 or microsomes, and this *in vitro* effect is due to the *in vivo* reduction of B[a]P hydroxylation activity in the microsomes by eugenol administration.

## Materials and methods

### Treatment of animals

Male Wistar rats (250 g) were used. Eugenol (200 mg) diluted to 1 ml with olive oil was administered orally 4 times to the rats at 12-h intervals and the rats were sacrificed 2 days after the final treatment. Controls received the same volume of olive oil concurrently. Phenobarbital (PB) was dissolved in saline and given in a daily intraperitoneal injection of 100 mg/kg and the rats were sacrificed 2 days after the final dose. 3-Methylcholanthrene (3-MC) and polychlorinated biphenyls (PCBs) were dissolved in olive oil and injected intraperitoneally as single doses of 80 and 500 mg/kg, respectively, and the rats were sacrificed on the 4th and 5th days, respectively. Dietary administration of eugenol to the rats was performed as follows. Male Wistar rats (6–7 weeks old) were obtained and housed in a room with a 12-h light-dark cycle. These rats had free access to a commercial diet and tap water during a week of acclimatization. Thereafter, a diet containing 5% (w/w) eugenol was fed to the experimental animals for 22 days. These rats were sacrificed on the 23rd day.

### Preparation of the S9 fraction and microsomes from rat liver

Rat liver, after being perfused with 0.15 M KCl, was minced and homogenated with 4 vol. of the same solution. The homogenate was centrifuged for 15 min at  $9000 \times g$ . This supernatant fraction is called the S9 fraction. Thereafter, this fraction was centrifuged at  $105000 \times g$  for 60 min to obtain the microsomes.

### Analytical procedures

The mutagenicity test using *Salmonella typhimurium* strain TA100 was carried out as described by Ames et al. [2], with a slight modification to allow use of the preincubation method. S9 mix (100 mM sodium phosphate buffer, pH 7.4, 8 mM

MgCl<sub>2</sub>, 33 mM KCl, 5 mM glucose 6-phosphate, 4 mM NADP and S9 protein), mutagen and test organisms were mixed and the mixture was incubated at 37°C. After 20 min the incubated mixture was mixed with 2 ml of soft agar and spread over a Vogel-Bonner agar plate. After 2-day incubation at 37°C, the number of revertants was counted. When microsomes were used instead of S9, 3.0 units/plate of glucose 6-phosphatase were added to the assay mixture. The data were always corrected for the background rate of reversion. All experiments were performed in triplicate. Arylhydrocarbon hydroxylase (AHH) was assayed by measurement of the production of 3-hydroxybenzo[a]pyrene as described elsewhere [19]. Total B[a]P hydroxylase was assayed by the direct fluorometric method described by Yang and Kitch [13]. Cytochrome P-450 was estimated as described by Omura and Sato [7]. Protein concentrations were determined by the method of Lowry et al. [6] using bovine serum albumin as the standard.

### Reagents and chemicals

Eugenol and B[a]P were obtained from Wako Pure Chemical Industries, Osaka, Japan. PB and 3-MC were from Sigma Chemicals Co., St. Louis, MO, U.S.A. The PCBs were a generous gift from Dr. C. Shimada, Public Health Institute of Osaka Prefecture.

## Results

### Suppressed mutagenicity of B[a]P

Fig. 1 shows the mutagenicity of B[a]P in the Ames test when the liver S9 fractions from the rats treated with eugenol or olive oil (control) were used. As positive controls, the S9 fractions from rats treated with PB, 3-MC and PCBs were also employed. It is well known that the mutagenicity of B[a]P is remarkably increased in the Ames test when 3-MC-treated or PCB-treated rat liver S9 fractions are used, while the mutagenicity is of the same level as that of untreated liver S9 fraction when PB-treated S9 fraction is used (Fig. 1A). We found that with use of eugenol-treated S9 fractions in the Ames test, the mutagenicity of B[a]P was suppressed to a lower level than that of the controls (Fig. 1A and B). This suppression of

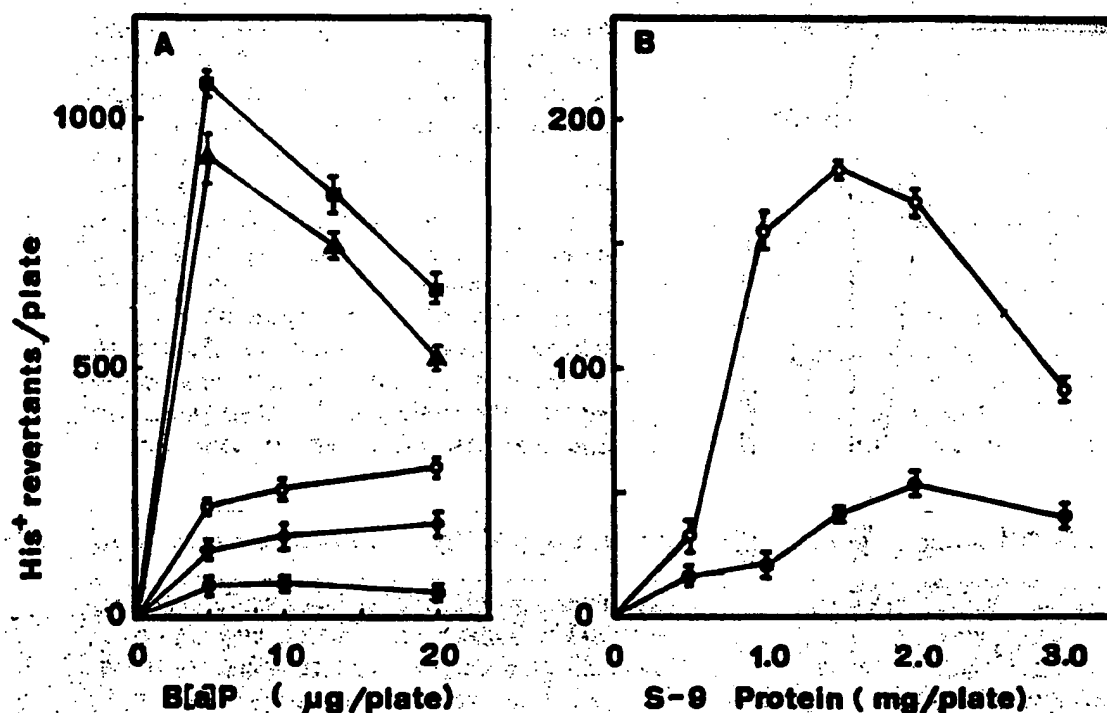


Fig. 1. Mutagenicity of B[a]P in the Ames test using liver S9 fractions from rats treated with eugenol (●), olive oil (○), PB (◆), 3-MC (■) and PCBs (▲). Appropriate amounts of B[a]P (A) and S9 protein (B) were added to the assay mixture. S9 protein (1.0 mg/plate) (A) and B[a]P (20 µg/plate) (B) were contained in the mixture and the assay was performed as described in Materials and Methods. All reversion values were corrected for spontaneous reversion frequency (about 100–130 revertant colonies). Each point is the average for 3 animals. Bars: S.E.

TABLE 1

EFFECTS OF EUGENOL ADDITION TO THE ASSAY MIXTURE ON MUTAGENICITY OF B[a]P AND ARYL-HYDROCARBON HYDROXYLASE ACTIVITY

The Ames test and enzyme assay of AHH were performed as described in Materials and Methods after addition of eugenol to the assay mixtures. In the Ames test, the amounts of B[a]P and microsomes from PCB-treated rat liver were 5 µg/plate and 0.1 mg/plate, respectively.

Eugenol addition (µM)	His <sup>+</sup> revertants per plate <sup>a</sup>	Arylhydrocarbon hydroxylase activity (nm/min/mg of protein)
0	667 ± 67	0.799 ± 0.066
10	622 ± 58	0.759 ± 0.023
50	631 ± 63	0.735 ± 0.015
100	801 ± 31	0.780 ± 0.034
1000	719 ± 82	0.806 ± 0.043

<sup>a</sup> Reversion values were corrected for spontaneous reversion frequency (about 100–130 revertant colonies). Means ± S.D. of 2 different experiments.

mutagenicity was also observed when microsomes, instead of the S9 fraction, from eugenol-treated rat liver were used as shown in Fig. 2. There was a reverse correlation between the mutagenicity of B[a]P and the dose of eugenol administered to the rats (Fig. 3). The mutagenicity of B[a]P was not suppressed by direct addition of eugenol into the S9 mix in the Ames test, and the activity of AHH, which catalyzed the reaction of B[a]P hydroxylation, was not inhibited either by the addition of eugenol in the reaction mixture (Table 1).

*Effects of eugenol administration on the activities of microsomal monooxygenase enzymes*

The effects of administration of eugenol on cytochrome P-450 content, AHH activity (which was the production rate of 3-hydroxybenzo[a]pyrene from B[a]P) and total B[a]P hydroxylase activity (which was the total conversion rate of

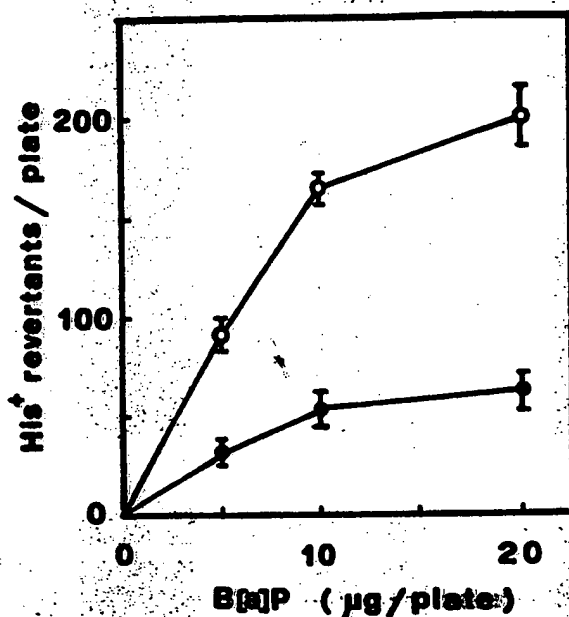


Fig. 2. Mutagenicity of B[a]P in the Ames test using liver microsomes from rats treated with eugenol (●) and an olive oil control (○). The data were obtained for appropriate amounts of B[a]P. The protein content of the microsomes was 0.3 mg/plate. All reversion values were corrected for spontaneous reversion frequency (about 100–130 revertant colonies). Each point is the average for 3 animals. Barr: S.E.

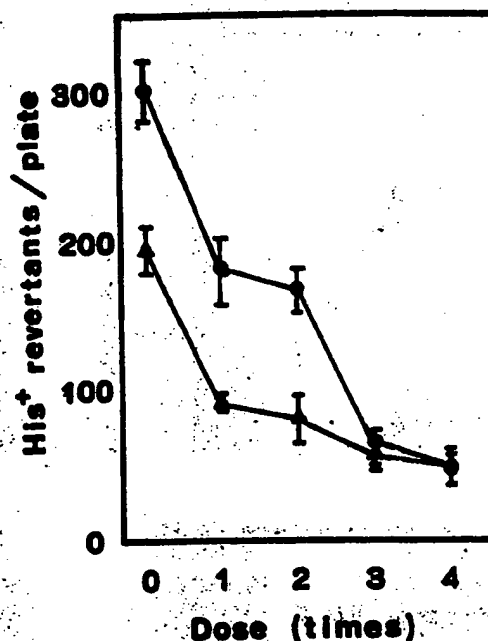


Fig. 3. Reverse correlation between the mutagenicity of B[a]P and the dose (200 mg × times) of eugenol administered to the rats. The amount of B[a]P was 20 μg/plate. The protein contents of the S9 fractions (●) and microsomes (Δ) were 1.0 and 0.3 mg/plate, respectively. All reversion values were corrected for spontaneous reversion frequency (about 100–130 revertant colonies). Each point is the average for 3 animals. Barr: S.E.

TABLE 2

EFFECTS OF ADMINISTRATION OF EUGENOL, 3-METHYLCHOLANTHRENE AND PHENOBARBITAL TO RATS ON CYTOCHROME P-450 CONTENT, ARYLHYDROCARBON HYDROXYLASE ACTIVITY AND THE RATE OF BENZO[a]PYRENE METABOLISM IN LIVER MICROSOMES

Eugenol (200 mg) was orally administered to the rats 4 times and other inducers were injected intraperitoneally as described in Materials and Methods. Cytochrome P-450 and enzyme activity were determined in the liver microsomes from various inducer-treated rats.

Inducers	P-450 content (nmoles/mg)	AHH activity (nmoles/min/mg)	Total B[a]P hydroxylase <sup>a</sup> (nmoles/min/mg)
Control	1.05 ± 0.05 (100) <sup>b</sup>	0.234 ± 0.045 (100)	0.103 ± 0.024 (100)
Eugenol	0.85 ± 0.02 (81)	0.067 ± 0.012 (29)	0.048 ± 0.006 (47)
3-Methylcholanthrene	1.37 ± 0.11 (130)	0.952 ± 0.184 (407)	3.923 ± 0.066 (5750)
Phenobarbital	2.18 ± 0.46 (208)	0.142 ± 0.022 (61)	0.110 ± 0.031 (107)

<sup>a</sup> These data were determined as described by Yang and Kitcha [13].

<sup>b</sup> % of control.

Means ± S.E. for 6 animals.

B[a]P to its metabolites) in the rat liver microsomes are shown in Table 2. In the 3-MC-treated microsomes, even when the total content of cytochrome P-450 was scarcely changed, AHH activity and total B[a]P hydroxylase activity were highly increased (Table 2). On the other hand, in spite of a 2-fold increase of cytochrome P-450 specific content, the specific activity of AHH per microsomal protein was decreased in 61% of the controls, and the specific activity of total B[a]P hydroxylase was not affected in PB-treated microsomes (Table 2). However, in PB-treated rat liver, the whole activities of AHH and B[a]P hydroxylase were increased, because the microsomal protein content per liver weight was greatly increased (data not shown). These data correspond with the facts that 3-MC induced the specific form of cytochrome P-450 which selectively catalyzed B[a]P to mutagens, and that PB induced the other form of cytochrome P-450 which catalyzed different precarcinogens from B[a]P, such as aflatoxin B<sub>1</sub> [4]. In the eugenol-treated rat liver microsomes, the content of cytochrome P-450 was decreased to 81% of the control value; furthermore, AHH activity and total B[a]P hydroxylase were decreased in 29 and 47% of the controls, respectively (Table 2).

#### Effects of dietary administration of eugenol

The contents of cytochrome P-450 and AHH

TABLE 3

EFFECTS OF ADMINISTRATION OF A DIET CONTAINING 5.0% (w/w) EUGENOL TO RATS ON CYTOCHROME P-450 CONTENT AND ARYLHYDROCARBON HYDROXYLASE ACTIVITY IN LIVER MICROSOMES, AND THE MUTAGENIC ACTIVITY OF B[a]P (5 µg/plate) IN THE AMES TEST USING RAT LIVER MICROSOMES

Diet	P-450 content (nmol/mg)	AHH activity (nmol/min/mg)	His <sup>+</sup> revertant colonies <sup>a</sup>
Control	0.853 ± 0.069 (100) <sup>b</sup>	0.189 ± 0.014 (100)	175 ± 21 (100)
Eugenol	0.815 ± 0.032 (96)	0.053 ± 0.010 (28)	15 ± 11 (9)

<sup>a</sup> Reversion values were corrected for spontaneous reversion frequency (about 100–130 revertant colonies).

<sup>b</sup> % of control.

Means ± S.E. for 5 animals.

activity in the liver microsomes from rats fed ad libitum on a diet containing 5% (w/w) eugenol for 22 days were about 68% of the control values as previously described [17]; the other data (liver weight, microsomal protein content per liver weight) were not changed by dietary administration of eugenol to the rats. In Table 3, it is shown that the dietary administration of eugenol to the rats decreased the content of cytochrome P-450 in the liver microsomes. The mutagenicity of B[a]P in the Ames test using the microsomes and AHH activity in the microsomes were also suppressed (Table 3).

#### Discussion

The suppressed mutagenicity of B[a]P (Figs. 1 and 2) was caused only by the eugenol administered to the rats, as can be seen in the results of the dose-response curve shown in Fig. 3. The microsomal components in the eugenol-treated S9 fraction seem to play an important role in this occurrence (Fig. 2). It has been reported that ellagic, chlorogenic, caffeic and ferulic acids [11], some chalcones [10] and aqueous and acetone extractions of some common vegetables [5] directly inhibit the mutagenic activity of B[a]P on *Salmonella typhimurium* when they are incubated in the assay plates. Such a direct inhibitory effect on B[a]P mutagenicity and significant effects on AHH activity were not observed in the case of eugenol in an in vitro assay (Table 1) previously described [17]. The mechanism of this suppressed mutagenicity by eugenol is different from that of the antimutagens described above, because their effects were in vitro while that of eugenol was in vivo. AHH activity would be the indicator of the mutagenic activity of B[a]P using liver microsomes from rats pretreated with two inducers (Table 2) as has been previously described [4,8,9]. Eugenol did not directly decrease AHH activity (Table 1). Therefore, suppression of the mutagenicity of B[a]P is obviously caused by the reduction of AHH activity mediated by cytochrome P-450 in eugenol-treated rat liver microsomes (Table 2), as in the observed event caused by 2(3)-tert-butyl-4-hydroxyanisole (BHA) [3].

Since the same suppression of mutagenicity and

reduction of AHH as described above were obtained in liver microsomes from the rats fed a diet containing 5% (w/w) eugenol for 22 days (Table 3), the activation of B[a]P to ultimate mutagen(s) or carcinogen(s) would be suppressed in the livers of rats fed the eugenol diet. Microsomal epoxide hydrolase activity in eugenol-treated microsomes was not observed in this study; however, this enzyme, which is known to catalyze a detoxification of activated B[a]P, B[a]P epoxides, is very interesting for further study of the suppression mechanism by eugenol. UDP-glucuronyltransferase could not play a role in detoxification of B[a]P in an Ames test, because UDP-glucuronic acid, which is a donor substrate of the enzyme, was not added to the assay mixture. In vivo, induction of the glucuronidation enzymes, cytosolic UDP-glucose dehydrogenase and microsomal UDP-glucuronyltransferase, as previously reported by us [14-16], and reduction of AHH would be effective in the removal of activated B[a]P. Since in vivo glucuronidation enzymes are fully active in rat liver containing UDP-glucuronic acid produced by UDP-glucose dehydrogenase, which is induced by eugenol, protective effects of eugenol in rat liver against the toxicity of carcinogenic compounds such as B[a]P would be expected, as were previously described for BHA of antimutagens [3] and anticarcinogens [11].

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## INHIBITION OF TUMOUR PROMOTION IN MICE BY EUGENOL

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( Received on March 15, 1993 )

**Abstract:** Number of tumours (papillomas) produced by the application of 7, 12-dimethyl benz (a) anthracene as initiator and croton oil promotor in mice were considerably inhibited (84%) by the prior application of eugenol. Moreover, there was considerable decrease in the number of tumour bearing animals and their onset. Eugenol inhibited superoxide formation and lipid peroxidation and the radical scavenging activity may be responsible for its chemopreventive action.

**Key words:** eugenol    tumour promotion    superoxides    lipid peroxidation

### INTRODUCTION

The concept of two-stage carcinogenesis consisting of initiation and promotion was first proposed by Berenblum (1). The former stage is an irreversible process while the latter is associated with reversible and irreversible changes, 12-O-Tetradecanoyl phorbol 13-acetate (TPA), present in croton oil is a typical tumour promotor having various biological and biochemical effects on susceptible tissues (2).

Recent studies have shown that several naturally occurring compounds exhibit anti-tumour promoting activity. These include quercetin, oleanolic acid, ursolic acid and kaempferol (3). Various spices like garlic, asafoetida and mace (4, 5) and curcumin the active ingredient present in turmeric (6,7), are also reported to possess this activity. Eugenol (4-allyl-2-methoxyphenol) is a naturally occurring compound which is used as a food flavour and fragrance agent (8). Eugenol is the main component of the oil of clove and is also present in the essential oils and in the extracts of many other plants including cinnamon basil and nutmeg (9).

In the present study, we have evaluated the effect of eugenol on croton oil-induced tumour promotion on mouse skin. Inhibitory effects of eugenol on superoxide production as well as lipid peroxidation were also studied.

### METHODS

Female Swiss albino mice (shaved on dorsal skin two days earlier), were divided into two groups of 10 animals each. They were initiated with single topical application of a solution of 470 nmol, of 7,12-dimethyl benz (a) anthracene (DMBA) in 200 µl acetone (7). After one week, control mice (Group I) received topical applications of croton oil (50 µl) as a promotor twice weekly for 6 weeks. Animals in Group II were also treated with Eugenol (2 mg in 0.1 ml acetone) 30-40 min prior to the application of promotor croton oil. Number of skin tumour (papilloma) formation on the mouse skin were recorded weekly and tumours greater than 1 mm in diameter were included in the cumulative total if they persisted two weeks or more. Values are mean of two independent experiments.

The lipid peroxidation was determined by the

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thiobarbituric acid (TBA) method (10). Mouse liver homogenate (25%, 100  $\mu$ l) in cold tris-HCl buffer (0.2M, pH 7.0) was incubated with and without eugenol for 1 hr at 37°C in the presence of 150 mM KCl (100  $\mu$ l), 0.3 mM ascorbic acid (100  $\mu$ l) 0.8 mM ferrous ammonium sulphate (100  $\mu$ l) in a total volume of 500  $\mu$ l. After incubation 20% trichloroacetic acid (1 ml) followed by 0.67% TBA (2 ml) were added to each tube and boiled for 15 min. After cooling and centrifugation at 200 g, the optical density of the supernatant was measured at 540 nm. The amount of lipid peroxidation was expressed as nmoles of malonaldehyde formed in each tube.

The scavenging effect of eugenol on photochemically-induced superoxide production was determined by the nitroblue tetrazolium (NBT) method (11). The assay mixture (3 ml) contained 0.1 M EDTA containing 0.0015% sodium cyanide (200  $\mu$ l), 1.5 mM NBT (100  $\mu$ l), 0.12 mM riboflavin (50  $\mu$ l) eugenol (different concentration) and phosphate buffer (pH 7.8). Optical densities were recorded before and after illuminations (15 min) at 560 nm. The difference in optical density in the control tubes and those containing eugenol was taken as a measure of superoxide production.

**Drugs :** Eugenol was purchased from Romali, India and 7,12-dimethyl benz (a) anthracene (DMBA) from Sigma Chemicals, St-Louis, USA. Croton oil was prepared from the seeds of *Croton tiglium* by petroleum ether extraction (4).

## RESULTS

In the control group, the first tumour appeared at week 5, whereas in the group treated with eugenol the first tumour appeared at week 12. Significant ( $P < 0.001$ ) inhibition of tumour formation was observed on the 16th week of initiation. Eugenol also lowered the percentage of tumour bearing mice (Fig.1). All the control animals developed tumours on 16th week of initiation while only 40% of the eugenol-treated group developed tumours at week 16. The average number of tumours per mouse at week 16 were  $2.56 \pm 0.78$  in the control group, and in the eugenol treated group  $0.4 \pm 0.50$  ( $P < 0.001$ ). Thus, treatment with eugenol caused 84% reduction in the average number of tumours per mouse at week 16.

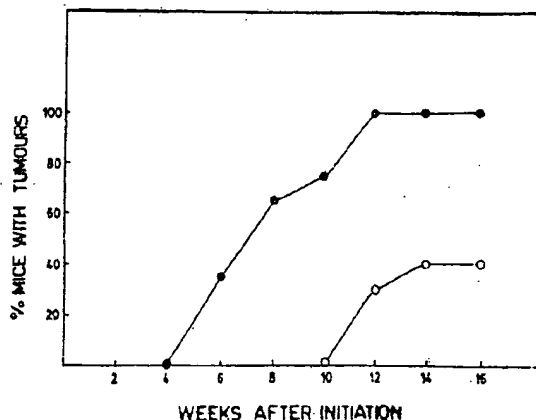


Fig. 1 : Effect of eugenol on papilloma formation.

The figure indicates the percentage of tumour bearing mice in ●—● control animals treated with DMBA and croton oil 0—○ animals treated with eugenol prior to croton oil application. Values are average of 2 independent experiments of 10 animals in each set.

Eugenol was found to inhibit superoxide production and lipid peroxidation in a significant manner ( $P < 0.001$ ) (Table I). The concentration required to produce 50% inhibition in the former was found to be 125  $\mu$ M, while the corresponding figure in the latter was 25  $\mu$ M.

TABLE I : Effect of eugenol on superoxide production and lipid peroxide formation *in vitro*.

Concentration of eugenol ( $\mu$ M)	Percentage inhibition	
	Superoxide production	Lipid peroxidation
25	0	28.7 $\pm$ 2.6
25	0	51.8 $\pm$ 3.4
50	12.5 $\pm$ 0.23	ND
100	39.6 $\pm$ 1.91	68.9 $\pm$ 5.2
500	79.2 $\pm$ 11.08	ND
1000	92.5 $\pm$ 10.2	89.4 $\pm$ 6.2

ND = not determined.

## DISCUSSION

It has been suggested that reactive oxygen species play an important role in tumour promotion (12). Free radical generating compounds such as

benzoyl peroxide, lauroyl peroxide and chloroper benzoic acid have tumour promoting activity in the mouse skin (6).

Eugenol has been reported to possess free radical scavenging activity (13). The present study revealed that eugenol inhibited *in vitro* superoxide production. Since superoxide dismutase (SOD), the metalloenzyme, which protect cells against oxygen mediated biological damage (14) is expressed at a low level during TPA-mediated tumour promotion, inhibition of superoxide production by eugenol assumes significance in reducing the tumour incidence. Lipid peroxidation products have been shown to cause considerable damage to DNA (15). In the present study, eugenol was found to inhibit *in vitro* lipid peroxidation. Moreover, we have shown that Eugenol could also

inhibit the chemical carcinogenesis induced by topical application of dimethyl benzanthrane followed by croton oil promotion. Because of the known free radical scavenging activity of eugenol it could be inferred that eugenol's action is mainly in the inhibition of promotion.

The effect of curcumin on TPA-induced tumour promotion has been attributed to the inhibition of arachidonic acid metabolism via the lipoxygenase and cyclo-oxygenase pathways, which result in the formation of reactive oxygen species and other free radicals (16). Eugenol has been reported to inhibit prostaglandin synthesis via promotion by inhibiting arachidonic acid metabolism and/or by functioning as a scavenger of reactive species that are produced during the metabolism of arachidonic acid.

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## PATENT COOPERATION TREATY

COPY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:

McDermott, Will & Emery  
Attn. GADIANO, WILLEM F  
600 13th Street, N.W.  
Washington DC 20005-3096  
UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT  
FOR THE DECLARATION

MAY 23 2000

(PCT Rule 44.1)

MW&amp;E

Date of mailing  
(day/month/year)

18/05/2000

Applicant's or agent's file reference

45112-046

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.

PCT/US 99/28889

International filing date  
(day/month/year)

07/12/1999

Applicant

ECOSMART TECHNOLOGIES, INC. et al.

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland  
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentaan 2  
NL-2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Nina Vercio

## NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

### INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

#### What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

#### When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

#### Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

#### How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

#### What documents must/may accompany the amendments?

##### Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

## NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

### "Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

**It must be in the language in which the international application is to be published.**

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

### Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

### Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>45112-046</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/US 99/ 28889</b>	International filing date (day/month/year) <b>07/12/1999</b>	(Earliest) Priority Date (day/month/year) <b>07/12/1998</b>
Applicant  <b>ECOSMART TECHNOLOGIES, INC. et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.



the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:



contained in the international application in written form.



filed together with the international application in computer readable form.



furnished subsequently to this Authority in written form.



furnished subsequently to this Authority in computer readable form.



the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.



the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2.



**Certain claims were found unsearchable** (See Box I).

3.



**Unity of invention is lacking** (see Box II).

4. With regard to the **title**,



the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,



the text is approved as submitted by the applicant.



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.



as suggested by the applicant.



because the applicant failed to suggest a figure.



because this figure better characterizes the invention.

1



None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 28889

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 15 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/28889

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 A61K35/78 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SYLVIE BARDON ET AL.: "MONOTERPENES INHIBIT CELL GROWTH, CELL CYCLE PROGRESSION, AND CYCLIN D1 GENE EXPRESSION IN HUMAN BREAST CANCER CELL LINES." NUTRITION AND CANCER, vol. 32, no. 1, 1998, pages 1-7, XP000892114 LONDON, GB ISSN: 0163-5581 the whole document	1,4,5
Y	GB 2 151 924 A (ROUSSEL UCLAF) 31 July 1985 (1985-07-31) page 1, line 62 -page 2, line 48	1,3
Y	FR 2 706 771 A (PELLETIER JACQUES) 30 December 1994 (1994-12-30) page 1, line 1 - line 47	1,3

☐

Further documents are listed in the continuation of box C.

☒

Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

11 May 2000

Date of mailing of the international search report

18/05/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Rempp, G

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/28889

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claim 15 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/28889

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		BE 901390 A	27-06-1985
		CH 661205 A	15-07-1987
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